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(54) Title: DNA SEQUENCING BY MASS SPECTROMETRY (57) Abstract <p>The invention describes a new method to sequence DNA. The improvements over the existing DNA sequencing technologies are high speed, high throughput, no electrophoresis and gel reading artifacts due to the complete absence of an electrophoretic step, and no costly reagents involving various substitutions with stable isotopes. The invention utilizes the Sanger sequencing strategy and assembles the sequence information by analysis of the nested fragments obtained by base-specific chain termination via their different molecular masses using mass spectrometry, as for example, MALDI or ES mass spectrometry. A further increase in throughput can be obtained by introducing mass-modifications in the oligonucleotide primer, chain-terminating nucleoside triphosphates and/or in the chain-elongating nucleoside triphosphates, as well as using integrated tag sequences which allow multiplexing by hybridization of tag specific probes with mass differentiated molecular weights.</p>		

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DNA SEQUENCING BY MASS SPECTROMETRY

Related Applications

This application is a continuation-in-part of U.S. Application Serial Number 08/617,010, which is a continuation-in-part of U.S. Application Serial Number 08/178,216, which issued as U.S. Patent No. 5,547,835, and which itself is a continuation-in-part of U.S. Application Serial Number 08/001,323 filed January 7, 1993, which is now abandoned. The contents of all related applications are incorporated herein by reference.

15

Background of the Invention

Since the genetic information is represented by the sequence of the four DNA building blocks deoxyadenosine- (dpA), deoxyguanosine- (dpG), deoxycytidine- (dpC) and deoxythymidine-5'-phosphate (dpT), DNA sequencing is one of the most fundamental technologies in molecular biology and the life sciences in general. The ease and the rate by which DNA sequences can be obtained greatly affects related technologies such as development and production of new therapeutic agents and new and useful varieties of plants and microorganisms via recombinant DNA technology. In particular, unraveling the DNA sequence helps in understanding human pathological conditions including genetic disorders, cancer and AIDS. In some cases, very subtle differences such as a one nucleotide deletion, addition or substitution can create serious, in some cases even fatal, consequences. Recently, DNA sequencing has become the core technology of the Human Genome Sequencing Project (e.g., J.F. Bishop and M. Waldholz, 1991, Genome: The Story of the Most Astonishing Scientific Adventure of Our Time - The Attempt to Map All the Genes in the Human Body, Simon & Schuster, New York). Knowledge of the complete human genome DNA sequence will certainly help to understand, to diagnose, to prevent and to treat human diseases. To be able to tackle successfully the determination of the approximately 3 billion base pairs of the human genome in a reasonable time frame and in an economical way, rapid, reliable,

sensitive and inexpensive methods need to be developed, which also offer the possibility of automation. The present invention provides such a technology

Recent reviews of today's methods together with future directions and trends are given by Barrell (The FASEB Journal **5**, 40-45 (1991)), and Trainor (Anal. Chem. **62**, 418-26 (1990)).

Currently, DNA sequencing is performed by either the chemical degradation method of Maxam and Gilbert (Methods in Enzymology **65**, 499-560 (1980)) or the enzymatic dideoxynucleotide termination method of Sanger *et al.* (Proc. Natl. Acad. Sci. USA **74**, 5463-67 (1977)). In the chemical method, base specific modifications result in a base specific cleavage of the radioactive or fluorescently labeled DNA fragment. With the four separate base specific cleavage reactions, four sets of nested fragments are produced which are separated according to length by polyacrylamide gel electrophoresis (PAGE). After autoradiography, the sequence can be read directly since each band (fragment) in the gel originates from a base specific cleavage event. Thus, the fragment lengths in the four "ladders" directly translate into a specific position in the DNA sequence.

In the enzymatic chain termination method, the four base specific sets of DNA fragments are formed by starting with a primer/template system elongating the primer into the unknown DNA sequence area and thereby copying the template and synthesizing a complementary strand by DNA polymerases, such as Klenow fragment of *E. coli* DNA polymerase I, a DNA polymerase from *Thermus aquaticus*, Taq DNA polymerase, or a modified T7 DNA polymerase, Sequenase (Tabor *et al.*, Proc. Natl. Acad. Sci. USA **84**, 4767-4771 (1987)), in the presence of chain-terminating reagents. Here, the chain-terminating event is achieved by incorporating into the four separate reaction mixtures in addition to the four normal deoxynucleoside triphosphates, dATP, dGTP, dTTP and dCTP, only one of the chain-terminating dideoxynucleoside triphosphates, ddATP, ddGTP, ddTTP or ddCTP, respectively, in a limiting small concentration. The four sets of resulting fragments produce, after electrophoresis, four base specific ladders from which the DNA sequence can be determined.

A recent modification of the Sanger sequencing strategy involves the degradation of phosphorothioate-containing DNA fragments obtained by using alpha-thio dNTP instead of the normally used ddNTPs during the primer extension reaction

mediated by DNA polymerase (Labeit *et al.*, DNA 5, 173-177 (1986); Amersham, PCT-Application GB86/00349; Eckstein *et al.*, Nucleic Acids Res. 16, 9947 (1988)). Here, the four sets of base-specific sequencing ladders are obtained by limited digestion with exonuclease III or snake venom phosphodiesterase, subsequent separation on PAGE and visualization by radioisotopic labeling of either the primer or one of the dNTPs. In a further modification, the base-specific cleavage is achieved by alkylating the sulphur atom in the modified phosphodiester bond followed by a heat treatment (Max-Planck-Gesellschaft, DE 3930312 A1). Both methods can be combined with the amplification of the DNA via the Polymerase Chain Reaction (PCR).

On the upfront end, the DNA to be sequenced has to be fragmented into sequencable pieces of currently not more than 500 to 1000 nucleotides. Starting from a genome, this is a multi-step process involving cloning and subcloning steps using different and appropriate cloning vectors such as YAC, cosmids, plasmids and M13 vectors (Sambrook *et al.*, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, 1989). Finally, for Sanger sequencing, the fragments of about 500 to 1000 base pairs are integrated into a specific restriction site of the replicative form I (RF I) of a derivative of the M13 bacteriophage (Vieria and Messing, Gene 19, 259 (1982)) and then the double-stranded form is transformed to the single-stranded circular form to serve as a template for the Sanger sequencing process having a binding site for a universal primer obtained by chemical DNA synthesis (Sinha, Biernat, McManus and Köster, Nucleic Acids Res. 12, 4539-57 (1984); U.S. Patent No. 4725677 upstream of the restriction site into which the unknown DNA fragment has been inserted. Under specific conditions, unknown DNA sequences integrated into supercoiled double-stranded plasmid DNA can be sequenced directly by the Sanger method (Chen and Seeburg, DNA 4, 165-170 (1985)) and Lim *et al.*, Gene Anal. Techn. 5, 32-39 (1988), and, with the Polymerase Chain Reaction (PCR) (PCR Protocols: A Guide to Methods and Applications, Innis *et al.*, editors, Academic Press, San Diego (1990)) cloning or subcloning steps could be omitted by directly sequencing off chromosomal DNA by first amplifying the DNA segment by PCR and then applying the Sanger sequencing method (Innis *et al.*, Proc. Natl. Acad. Sci. USA 85, 9436-9440 (1988)). In this case, however, the DNA sequence in the interested region must be known at least to the extent to bind a sequencing primer.

In order to be able to read the sequence from PAGE, detectable labels have to be used in either the primer (very often at the 5'-end) or in one of the deoxynucleoside triphosphates, dNTP. Using radioisotopes such as ^{32}P , ^{33}P , or ^{35}S is still the most frequently used technique. After PAGE, the gels are exposed to X-ray films and silver grain exposure is analyzed. The use of radioisotopic labeling creates several problems. Most labels useful for autoradiographic detection of sequencing fragments have relatively short half-lives which can limit the useful time of the labels. The emission high energy beta radiation, particularly from ^{32}P , can lead to breakdown of the products via radiolysis so that the sample should be used very quickly after labeling. In addition, high energy radiation can also cause a deterioration of band sharpness by scattering. Some of these problems can be reduced by using the less energetic isotopes such as ^{33}P or ^{35}S (see, e.g., Ornstein *et al.*, Biotechniques 3, 476 (1985)). Here, however, longer exposure times have to be tolerated. Above all, the use of radioisotopes poses significant health risks to the experimentalist and, in heavy sequencing projects, decontamination and handling the radioactive waste are other severe problems and burdens.

In response to the above mentioned problems related to the use of radioactive labels, non-radioactive labeling techniques have been explored and, in recent years, integrated into partly automated DNA sequencing procedures. All these improvements utilize the Sanger sequencing strategy. The fluorescent label can be tagged to the primer (Smith *et al.*, Nature 321, 674-679 (1986) and EPO Patent No. 87300998.9; Du Pont De Nemours EPO Application No. 0359225; Ansorge *et al.* L. Biochem. Biophys. Methods 13, 325-32 (1986)) or to the chain-terminating dideoxynucleoside triphosphates (Prober *et al.* Science 238, 336-41 (1987); Applied Biosystems, PCT Application WO 91/05060). Based on either labeling the primer or the ddNTP, systems have been developed by Applied Biosystems (Smith *et al.*, Science 235, G89 (1987); U.S. Patent Nos. 570973 and 689013), Du Pont De Nemours (Prober *et al.* Science 238, 336-341 (1987); U.S. Patents Nos. 881372 and 57566), Pharmacia-LKB (Ansorge *et al.* Nucleic Acids Res. 15, 4593-4602 (1987) and EMBL Patent Application DE P3724442 and P3805808.1) and Hitachi (JP 1-90844 and DE 4011991 A1). A somewhat similar approach was developed by Brumbaugh *et al.* (Proc. Natl. Sci. USA 85, 5610-14 (1988) and U.S. Patent No. 4,729,947). An improved method for the Du Pont system using two electrophoretic lanes with two different specific labels per lane is

described (PCT Application WO92/02635). A different approach uses fluorescently labeled avidin and biotin labeled primers. Here, the sequencing ladders ending with biotin are reacted during electrophoresis with the labeled avidin which results in the detection of the individual sequencing bands (Brumbaugh *et al.*, U.S. Patent No. 594676).

5 More recently even more sensitive non-radioactive labeling techniques for DNA using chemiluminescence triggerable and amplifiable by enzymes have been developed (Beck, O'Keefe, Coull and Köster, Nucleic Acids Res. 17, 5115-5123 (1989) and Beck and Köster, Anal. Chem. 62, 2258-2270 (1990)). These labeling methods were combined with multiplex DNA sequencing (Church *et al.* Science 240, 185-188 (1988) to
10 provide for a strategy aimed at high throughput DNA sequencing (Köster *et al.*, Nucleic Acids Res. Symposium Ser. No. 24, 318-321 (1991), University of Utah, PCT Application No. WO 90/15883); this strategy still suffers from the disadvantage of being very laborious and difficult to automate.

In an attempt to simplify DNA sequencing, solid supports have been
15 introduced. In most cases published so far, the template strand for sequencing (with or without PCR amplification) is immobilized on a solid support most frequently utilizing the strong biotin-avidin/streptavidin interaction (Orion-Yhtymä Oy, U.S. Patent No. 277643; M. Uhlen *et al.* Nucleic Acids Res. 16, 3025-38 (1988); Cemü Bioteknik, PCT Application No. WO 89/09282 and Medical Research Council, GB, PCT Application No.
20 WO 92/03575). The primer extension products synthesized on the immobilized template strand are purified of enzymes, other sequencing reagents and by-products by a washing step and then released under denaturing conditions by loosing the hydrogen bonds between the Watson-Crick base pairs and subjected to PAGE separation. In a different approach, the primer extension products (not the template) from a DNA sequencing
25 reaction are bound to a solid support via biotin/avidin (Du Pont De Nemours, PCT Application WO 91/11533). In contrast to the above mentioned methods, here, the interaction between biotin and avidin is overcome by employing denaturing conditions (formamide/EDTA) to release the primer extension products of the sequencing reaction from the solid support for PAGE separation. As solid supports, beads, (e.g., magnetic
30 beads (Dynabeads) and Sepharose beads), filters, capillaries, plastic dipsticks (e.g., polystyrene strips) and microtiter wells are being proposed.

All methods discussed so far have one central step in common: polyacrylamide gel electrophoresis (PAGE). In many instances, this represents a major drawback and limitation for each of these methods. Preparing a homogeneous gel by polymerization, loading of the samples, the electrophoresis itself, detection of the sequence pattern (e.g., by autoradiography), removing the gel and cleaning the glass plates to prepare another gel are very laborious and time-consuming procedures. Moreover, the whole process is error-prone, difficult to automate, and, in order to improve reproducibility and reliability, highly trained and skilled personnel are required. In the case of radioactive labeling, autoradiography itself can consume from hours to days. In the case of fluorescent labeling, at least the detection of the sequencing bands is being performed automatically when using the laser-scanning devices integrated into commercial available DNA sequencers. One problem related to the fluorescent labeling is the influence of the four different base-specific fluorescent tags on the mobility of the fragments during electrophoresis and a possible overlap in the spectral bandwidth of the four specific dyes reducing the discriminating power between neighboring bands, hence, increasing the probability of sequence ambiguities. Artifacts are also produced by base-specific interactions with the polyacrylamide gel matrix (Frank and Köster, Nucleic Acids Res. 6, 2069 (1979)) and by the formation of secondary structures which result in "band compressions" and hence do not allow one to read the sequence. This problem has, in part, been overcome by using 7-deazadeoxyguanosine triphosphates (Barr *et al.*, Biotechniques 4, 428 (1986)). However, the reasons for some artifacts and conspicuous bands are still under investigation and need further improvement of the gel electrophoretic procedure.

A recent innovation in electrophoresis is capillary zone electrophoresis (CZE) (Jorgenson *et al.*, J. Chromatography 352, 337 (1986); Gesteland *et al.*, Nucleic Acids Res. 18, 1415-1419 (1990)) which, compared to slab gel electrophoresis (PAGE), significantly increases the resolution of the separation, reduces the time for an electrophoretic run and allows the analysis of very small samples. Here, however, other problems arise due to the miniaturization of the whole system such as wall effects and the necessity of highly sensitive on-line detection methods. Compared to PAGE, another drawback is created by the fact that CZE is only a "one-lane" process, whereas in PAGE samples in multiple lanes can be electrophoresed simultaneously.

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Due to the severe limitations and problems related to having PAGE as an integral and central part in the standard DNA sequencing protocol, several methods have been proposed to do DNA sequencing without an electrophoretic step. One approach calls for hybridization or fragmentation sequencing (Bains, Biotechnology 10, 757-58 (1992) and Mirzabekov *et al.*, FEBS Letters 256, 118-122 (1989)) utilizing the specific hybridization of known short oligonucleotides (e.g., octadeoxynucleotides which gives 65,536 different sequences) to a complementary DNA sequence. Positive hybridization reveals a short stretch of the unknown sequence. Repeating this process by performing hybridizations with all possible octadeoxynucleotides should theoretically determine the sequence. In a completely different approach, rapid sequencing of DNA is done by unilaterally degrading one single, immobilized DNA fragment by an exonuclease in a moving flow stream and detecting the cleaved nucleotides by their specific fluorescent tag via laser excitation (Jett *et al.*, J. Biomolecular Structure & Dynamics 7, 301-309, (1989); United States Department of Energy, PCT Application No. WO 89/03432). In another system proposed by Hyman (Anal. Biochem. 174, 423-436 (1988)), the pyrophosphate generated when the correct nucleotide is attached to the growing chain on a primer-template system is used to determine the DNA sequence. The enzymes used and the DNA are held in place by solid phases (DEAE-Sepharose and Sepharose) either by ionic interactions or by covalent attachment. In a continuous flow-through system, the amount of pyrophosphate is determined via bioluminescence (luciferase). A synthesis approach to DNA sequencing is also used by Tsien *et al.* (PCT Application No. WO 91/06678). Here, the incoming dNTP's are protected at the 3'-end by various blocking groups such as acetyl or phosphate groups and are removed before the next elongation step, which makes this process very slow compared to standard sequencing methods. The template DNA is immobilized on a polymer support. To detect incorporation, a fluorescent or radioactive label is additionally incorporated into the modified dNTP's. The same patent application also describes an apparatus designed to automate the process.

Mass spectrometry, in general, provides a means of "weighing" individual molecules by ionizing the molecules *in vacuo* and making them "fly" by volatilization. Under the influence of combinations of electric and magnetic fields, the ions follow

trajectories depending on their individual mass (m) and charge (z). In the range of molecules with low molecular weight, mass spectrometry has long been part of the routine physical-organic repertoire for analysis and characterization of organic molecules by the determination of the mass of the parent molecular ion. In addition, by arranging collisions of this parent molecular ion with other particles (e.g., argon atoms), the molecular ion is fragmented forming secondary ions by the so-called collision induced dissociation (CID). The fragmentation pattern/pathway very often allows the derivation of detailed structural information. Many applications of mass spectrometric methods in the known in the art, particularly in biosciences, and can be found summarized in Methods in Enzymology, Vol. 193: "Mass Spectrometry" (J.A. McCloskey, editor), 1990, Academic Press, New York

Due to the apparent analytical advantages of mass spectrometry in providing high detection sensitivity, accuracy of mass measurements, detailed structural information by CID in conjunction with an MS/MS configuration and speed, as well as on-line data transfer to a computer, there has been considerable interest in the use of mass spectrometry for the structural analysis of nucleic acids. Recent reviews summarizing this field include K. H. Schram, "Mass Spectrometry of Nucleic Acid Components, Biomedical Applications of Mass Spectrometry" 34, 203-287 (1990); and P.F. Crain, "Mass Spectrometric Techniques in Nucleic Acid Research," Mass Spectrometry Reviews 9, 505-554 (1990). The biggest hurdle to applying mass spectrometry to nucleic acids is the difficulty of volatilizing these very polar biopolymers. Therefore, "sequencing" has been limited to low molecular weight synthetic oligonucleotides by determining the mass of the parent molecular ion and through this, confirming the already known sequence, or alternatively, confirming the known sequence through the generation of secondary ions (fragment ions) via CID in an MS/MS configuration utilizing, in particular, for the ionization and volatilization, the method of fast atomic bombardment (FAB mass spectrometry) or plasma desorption (PD mass spectrometry). As an example, the application of FAB to the analysis of protected dimeric blocks for chemical synthesis of oligodeoxynucleotides has been described (Köster *et al.* Biomedical Environmental Mass Spectrometry 14, 111-116 (1987)).

Two more recent ionization/desorption techniques are electrospray/ion spray (ES) and matrix-assisted laser desorption/ionization (MALDI). ES mass spectrometry has been introduced by Fenn *et al.* (J. Phys. Chem. **88**, 4451-59 (1984); PCT Application No. WO 90/14148) and current applications are summarized in recent review articles (R.D. Smith *et al.*, Anal. Chem. **62**, 882-89 (1990) and B. Ardrey, Electrospray Mass Spectrometry, Spectroscopy Europe, **4**, 10-18 (1992)). The molecular weights of the tetradecanucleotide d(CATGCCATGGCATG) (SEQ ID NO:1) (Covey *et al.* "The Determination of Protein, Oligonucleotide and Peptide Molecular Weights by Ion spray Mass Spectrometry," Rapid Communications in Mass Spectrometry, **2**, 249-256 (1988)), of the 21-mer d(AAATTGTGCACATCCTGCAGC) (SEQ ID NO:2) and without giving details of that of a tRNA with 76 nucleotides (Methods in Enzymology, **193**, "Mass Spectrometry" (McCloskey, editor), p. 425, 1990, Academic Press, New York) have been published. As a mass analyzer, a quadrupole is most frequently used. The determination of molecular weights in femtomole amounts of sample is very accurate due to the presence of multiple ion peaks which all could be used for the mass calculation.

MALDI mass spectrometry, in contrast, can be particularly attractive when a time-of-flight (TOF) configuration is used as a mass analyzer. The MALDI-TOF mass spectrometry has been introduced by Hillenkamp *et al.* ("Matrix Assisted UV-Laser Desorption/Ionization: A New Approach to Mass Spectrometry of Large Biomolecules," Biological Mass Spectrometry (Burlingame and McCloskey, editors), Elsevier Science Publishers, Amsterdam, pp. 49-60, 1990.) Since, in most cases, no multiple molecular ion peaks are produced with this technique, the mass spectra, in principle, look simpler compared to ES mass spectrometry. Although DNA molecules up to a molecular weight of 410,000 daltons could be desorbed and volatilized (Williams *et al.*, "Volatilization of High Molecular Weight DNA by Pulsed Laser Ablation of Frozen Aqueous Solutions," Science, **246**, 1585-87 (1989)), this technique has so far only been used to determine the molecular weights of relatively small oligonucleotides of known sequence, e.g., oligothymidylic acids up to 18 nucleotides (Huth-Fehre *et al.*, "Matrix-Assisted Laser Desorption Mass Spectrometry of Oligodeoxythymidylic Acids," Rapid Communications in Mass Spectrometry, **6**, 209-13 (1992)) and a double-stranded DNA of 28 base pairs (Williams *et al.*, "Time-of-Flight Mass Spectrometry of Nucleic

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Acids by Laser Ablation and Ionization from a Frozen Aqueous Matrix," Rapid Communications in Mass Spectrometry, 4, 348-351 (1990)). In one publication (Huth-Fehre *et al.*, 1992, *supra*), it was shown that a mixture of all the oligothymidylic acids from $n=12$ to $n=18$ nucleotides could be resolved.

5 In U.S. Patent No. 5,064,754, RNA transcripts extended by DNA both of which are complementary to the DNA to be sequenced are prepared by incorporating NTP's, dNTP's and, as terminating nucleotides, ddNTP's which are substituted at the 5'-position of the sugar moiety with one or a combination of the isotopes ^{12}C , ^{13}C , ^{14}C , ^1H , ^2H , ^3H , ^{16}O , ^{17}O and ^{18}O . The polynucleotides obtained are degraded to 3'-
10 nucleotides, cleaved at the N-glycosidic linkage and the isotopically labeled 5'-functionality removed by periodate oxidation and the resulting formaldehyde species determined by mass spectrometry. A specific combination of isotopes serves to discriminate base-specifically between internal nucleotides originating from the incorporation of NTP's and dNTP's and terminal nucleotides caused by linking ddNTP's
15 to the end of the polynucleotide chain. A series of RNA/DNA fragments is produced, and in one embodiment, separated by electrophoresis, and, with the aid of the so-called matrix method of analysis, the sequence is deduced.

In Japanese Patent No. 59-131909, an instrument is described which detects nucleic acid fragments separated either by electrophoresis, liquid chromatography or high
20 speed gel filtration. Mass spectrometric detection is achieved by incorporating into the nucleic acids atoms which normally do not occur in DNA such as S, Br, I or Ag, Au, Pt, Os, Hg. The method, however, is not applied to sequencing of DNA using the Sanger method. In particular, it does not propose a base-specific correlation of such elements to an individual ddNTP.

25 PCT Application No. WO 89/12694 (Brennan *et al.*, Proc. SPIE-Int. Soc. Opt. Eng. 1206, (New Technol. Cytom. Mol. Biol.), pp. 60-77 (1990); and Brennan, U.S. Patent No. 5,003,059) employs the Sanger methodology for DNA sequencing by using a combination of either the four stable isotopes ^{32}S , ^{33}S , ^{34}S , ^{36}S or ^{35}Cl , ^{37}Cl , ^{79}Br , ^{81}Br to specifically label the chain-terminating ddNTP's. The sulfur isotopes can
30 be located either in the base or at the alpha-position of the triphosphate moiety whereas the halogen isotopes are located either at the base or at the 3'-position of the sugar ring

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The sequencing reaction mixtures are separated by an electrophoretic technique such as CZE, transferred to a combustion unit in which the sulfur isotopes of the incorporated ddNTP's are transformed at about 900°C in an oxygen atmosphere. The SO₂ generated with masses of 64, 65, 66 or 68 is determined on-line by mass spectrometry using, e.g., as
5 mass analyzer, a quadrupole with a single ion-multiplier to detect the ion current.

A similar approach is proposed in U.S. Patent No. 5,002,868 (Jacobson *et al.*, Proc. SPIE-Int. Soc. Opt. Eng. 1435, (Opt. Methods Ultrasensitive Detect. Anal. Tech. Appl.), 26-35 (1991)) using Sanger sequencing with four ddNTP's specifically substituted at the alpha-position of the triphosphate moiety with one of the four stable
10 sulfur isotopes as described above and subsequent separation of the four sets of nested sequences by tube gel electrophoresis. The only difference is the use of resonance ionization spectroscopy (RIS) in conjunction with a magnetic sector mass analyzer as disclosed in U.S. Patent No. 4,442,354 to detect the sulfur isotopes corresponding to the specific nucleotide terminators, and by this, allowing the assignment of the DNA
15 sequence.

EPO Patent Applications No. 0360676 A1 and 0360677 A1 also describe Sanger sequencing using stable isotope substitutions in the ddNTP's such as D, ¹³C, ¹⁵N, ¹⁷O, ¹⁸O, ³²S, ³³S, ³⁴S, ³⁶S, ¹⁹F, ³⁵Cl, ³⁷Cl, ⁷⁹Br, ⁸¹Br and ¹²⁷I or functional groups such as CF₃ or Si(CH₃)₃ at the base, the sugar or the alpha position of the
20 triphosphate moiety according to chemical functionality. The Sanger sequencing reaction mixtures are separated by tube gel electrophoresis. The effluent is converted into an aerosol by the electrospray/thermospray nebulizer method and then atomized and ionized by a hot plasma (7000 to 8000°K) and analyzed by a simple mass analyzer. An instrument is proposed which enables one to automate the analysis of the Sanger
25 sequencing reaction mixture consisting of tube electrophoresis, a nebulizer and a mass analyzer.

The application of mass spectrometry to perform DNA sequencing by the hybridization/fragment method (see above) has been recently suggested (Bains, "DNA Sequencing by Mass Spectrometry: Outline of a Potential Future Application,"
30 Chimicaoggi 2, 13-16 (1991)).

Summary of the Invention

The invention describes a new method to sequence DNA. The improvements over the existing DNA sequencing technologies include high speed, high throughput, no required electrophoresis (and, thus, no gel reading artifacts due to the complete absence of an electrophoretic step), and no costly reagents involving various substitutions with stable isotopes. The invention utilizes the Sanger sequencing strategy and assembles the sequence information by analysis of the nested fragments obtained by base-specific chain termination via their different molecular masses using mass spectrometry, for example, MALDI or ES mass spectrometry. A further increase in throughput can be obtained by introducing mass modifications in the oligonucleotide primer, the chain-terminating nucleoside triphosphates and/or the chain-elongating nucleoside triphosphates, as well as using integrated tag sequences which allow multiplexing by hybridization of tag specific probes with mass differentiated molecular weights.

Brief Description of the FIGURES

FIGURE 1 is a representation of a process to generate the samples to be analyzed by mass spectrometry. This process entails insertion of a DNA fragment of unknown sequence into a cloning vector such as derivatives of M13, pUC or phagemids; transforming the double-stranded form into the single-stranded form; performing the four Sanger sequencing reactions; linking the base-specifically terminated nested fragment family temporarily to a solid support; removing by a washing step all by-products; conditioning the nested DNA or RNA fragments by, for example, cation-ion exchange or modification reagent and presenting the immobilized nested fragments either directly to mass spectrometric analysis or cleaving the purified fragment family off the support and evaporating the cleavage reagent.

FIGURE 2A shows the Sanger sequencing products using ddTTP as terminating deoxynucleoside triphosphate of a hypothetical DNA fragment of 50 nucleotides (SEQ ID NO:3) in length with approximately equally balanced base composition. The molecular masses of the various chain terminated fragments are given.

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FIGURE 2B shows an idealized mass spectrum of such a DNA fragment mixture.

FIGURES 3A and 3B show, in analogy to FIGURES 2A and 2B, data for the same model sequence (SEQ ID NO:3) with ddATP as chain terminator.

5 FIGURES 4A and 4B show data, analogous to FIGURES 2A and 2B when ddGTP is used as a chain terminator for the same model sequence (SEQ ID NO:3).

 FIGURES 5A and 5B illustrate the results obtained where chain termination is performed with ddCTP as a chain terminator, in a similar way as shown in FIGURES 2A and 2B for the same model sequence (SEQ ID NO:3).

10 FIGURE 6 summarizes the results of FIGURES 2A to 5B, showing the correlation of molecular weights of the nested four fragment families to the DNA sequence (SEQ ID NO:3).

 FIGURE 7 illustrates the general structure of mass-modified sequencing nucleic acid primers or tag sequencing probes for either Sanger DNA or Sanger RNA sequencing.

15 FIGURE 8 shows the general structure for the mass-modified triphosphates for either Sanger DNA or Sanger RNA sequencing. General formulas of the chain-elongating and the chain-terminating nucleoside triphosphates are demonstrated.

20 FIGURE 9 outlines various linking chemistries (X) with either polyethylene glycol or terminally monoalkylated polyethylene glycol (R) as an example.

 FIGURE 10 illustrates similar linking chemistries as shown in FIGURE 8 and depicts various mass modifying moieties (R).

25 FIGURE 11 outlines how multiplex mass spectrometric sequencing can work using the mass-modified nucleic acid primer (UP).

 FIGURE 12 shows the process of multiplex mass spectrometric sequencing employing mass-modified chain-elongating and/or terminating nucleoside triphosphates.

30 FIGURE 13 shows multiplex mass spectrometric sequencing by involving the hybridization of mass-modified tag sequence specific probes.

FIGURE 14 shows a MALDI-TOF spectrum of a mixture of oligothymidylic acids, d(pT)₁₂₋₁₈.

FIGURE 15 shows a superposition of MALDI-TOF spectra of the 50-mer d(TAACGGTCATTACGGCCATTGACTGTAGGACCTGCATTACATGACTAGCT) (SEQ ID NO:3) (500 fmol) and dT(pdT)₉₉ (500 fmol).

FIGURE 16 shows the MALDI-TOF spectra of all 13 DNA sequences representing the nested dT-terminated fragments of the Sanger DNA sequencing simulation of Figure 2, 500 fmol each.

FIGURE 17 shows the superposition of the spectra of FIGURE 16. The two panels show two different scales and the spectra analyzed at that scale

FIGURE 18 shows the superimposed MALDI-TOF spectra from MALDI-MS analysis of mass-modified oligonucleotides as described in Example 21.

FIGURE 19 illustrates various linking chemistries between the solid support (P) and the nucleic acid primer (NA) through a strong electrostatic interaction.

FIGURE 20 illustrates various linking chemistries between the solid support (P) and the nucleic acid primer (NA) through a charge transfer complex of a charge transfer acceptor (A) and a charge transfer donor (D).

FIGURE 21 illustrates various linking chemistries between the solid support (P) and the nucleic acid primer (NA) through a stable organic radical.

FIGURE 22 illustrates a possible linking chemistry between the solid support (P) and the nucleic acid primer (NA) through Watson-Crick base pairing.

FIGURE 23 illustrates linking the solid support (P) and the nucleic acid primer (NA) through a photolytically cleavable bond.

FIGURE 24 shows the portion of the sequence of pRFc1 DNA, which was used as template for PCR amplification of unmodified and 7-deazapurine containing 99-mer and 200-mer nucleic acids as well as the sequences of the 19-mer primers and the two 18-mer reverse primers

FIGURE 25 shows the portion of the nucleotide sequence of M13mp18 RFI DNA, which was used for PCR amplification of unmodified and 7-deazapurine containing 103-mer nucleic acids. Also shown are nucleotide sequences of the 17-mer primers used in the PCR.

FIGURE 26 shows the result of a polyacrylamide gel electrophoresis of PCR products purified and concentrated for MALDI-TOF MS analysis. M: chain length marker, lane 1: 7-deazapurine containing 99-mer PCR product, lane 2: unmodified 99-mer, lane 3: 7-deazapurine containing 103-mer and lane 4: unmodified 103-mer PCR product.

FIGURE 27: an autoradiogram of polyacrylamide gel electrophoresis of PCR reactions carried out with 5'-[³²P]-labeled primers 1 and 4. Lanes 1 and 2: unmodified and 7-deazapurine modified 103-mer PCR product (53321 and 23520 counts), lanes 3 and 4: unmodified and 7-deazapurine modified 200-mer (71123 and 39582 counts) and lanes 5 and 6: unmodified and 7-deazapurine modified 99-mer (173216 and 94400 counts).

FIGURE 28: a) MALDI-TOF mass spectrum of the unmodified 103-mer PCR products (sum of twelve single shot spectra). The mean value of the masses calculated for the two single strands (31768 u and 31759 u) is 31763 u. Mass resolution: 18. b) MALDI-TOF mass spectrum of 7-deazapurine containing 103-mer PCR product (sum of three single shot spectra). The mean value of the masses calculated for the two single strands (31727 u and 31719 u) is 31723 u. Mass resolution: 67.

FIGURE 29: a) MALDI-TOF mass spectrum of the unmodified 99-mer PCR product (sum of twenty single shot spectra). Values of the masses calculated for the two single strands: 30261 u and 30794 u. b) MALDI-TOF mass spectrum of the 7-deazapurine containing 99-mer PCR product (sum of twelve single shot spectra). Values of the masses calculated for the two single strands: 30224 u and 30750 u.

FIGURE 30: a) MALDI-TOF mass spectrum of the unmodified 200-mer PCR product (sum of 30 single shot spectra). The mean value of the masses calculated for the two single strands (61873 u and 61595 u) is 61734 u. Mass resolution: 28. b) MALDI-TOF mass spectrum of 7-deazapurine containing 200-mer PCR product (sum of 30 single shot spectra). The mean value of the masses calculated for the two single strands (61772 u and 61514 u) is 61643 u. Mass resolution: 39.

FIGURE 31: a) MALDI-TOF mass spectrum of 7-deazapurine containing 100-mer PCR product with ribomodified primers. The mean value of the masses calculated for the two single strands (30529 u and 31095 u) is 30812 u. b) MALDI-TOF

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mass spectrum of the PCR-product after hydrolytic primer-cleavage. The mean value of the masses calculated for the two single strands (25104 u and 25229 u) is 25167 u. The mean value of the cleaved primers (5437 u and 5918 u) is 5677 u.

5 FIGURE 32 A-D shows the MALDI-TOF mass spectrum of the four sequencing ladders obtained from a 39-mer template (SEQ. ID. No. 13), which was immobilized to streptavidin beads via a 3' biotinylation. A 14-mer primer (SEQ. ID. NO. 14) was used in the sequencing.

10 FIGURE 33 shows a MALDI-TOF mass spectrum of a solid state sequencing of a 78-mer template (SEQ. ID. No. 15), which was immobilized to streptavidin beads via a 3' biotinylation. A 18-mer primer (SEQ ID No 16) and ddGTP were used in the sequencing.

FIGURE 34 shows a scheme in which duplex DNA probes with single-stranded overhang capture specific DNA templates and also serve as primers for solid state sequencing.

15 FIGURE 35A-D shows MALDI-TOF mass spectra obtained from a 5' fluorescent labeled 23-mer (SEQ. ID. No. 19) annealed to an 3' biotinylated 18-mer (SEQ. ID. No. 20), leaving a 5-base overhang, which captured a 15-mer template (SEQ ID. No. 21).

20 FIGURE 36 shows a stacking fluoroqram of the same products obtained from the reaction described in FIGURE 35, but run on a conventional DNA sequencer.

Detailed Description of the Invention

25 This invention describes an improved method of sequencing DNA. In particular, this invention employs mass spectrometry to analyze the Sanger sequencing reaction mixtures.

In Sanger sequencing, four families of chain-terminated fragments are obtained. The mass difference per nucleotide addition is 289.19 for dpC, 313.21 for dpA, 329.21 for dpG and 304.2 for dpT, respectively.

30 In one embodiment, through the separate determination of the molecular weights of the four base-specifically terminated fragment families, the DNA sequence can be assigned via superposition (e.g., interpolation) of the molecular weight peaks of the

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four individual experiments. In another embodiment, the molecular weights of the four specifically terminated fragment families can be determined simultaneously by MS, either by mixing the products of all four reactions run in at least two separate reaction vessels (i.e., all run separately, or two together, or three together) or by running one reaction
5 having all four chain-terminating nucleotides (e.g., a reaction mixture comprising dTTP, ddTTP, dATP, ddATP, dCTP, ddCTP, dGTP, ddGTP) in one reaction vessel. By simultaneously analyzing all four base-specifically terminated reaction products, the molecular weight values have been, in effect, interpolated. Comparison of the mass difference measured between fragments with the known masses of each chain-terminating
10 nucleotide allows the assignment of sequence to be carried out. In some instances, it may be desirable to mass modify, as discussed below, the chain-terminating nucleotides so as to expand the difference in molecular weight between each nucleotide. It will be apparent to those skilled in the art when mass-modification of the chain-terminating nucleotides is desirable and can depend, for instance, on the resolving ability of the particular
15 spectrometer employed. By way of example, it may be desirable to produce four chain-terminating nucleotides, ddTTP, ddCTP¹, ddATP² and ddGTP³ where ddCTP¹, ddATP² and ddGTP³ have each been mass-modified so as to have molecular weights resolvable from one another by the particular spectrometer being used.

The terms chain-elongating nucleotides and chain-terminating nucleotides
20 are well known in the art. For DNA, chain-elongating nucleotides include 2'-deoxyribonucleotides and chain-terminating nucleotides include 2', 3'-dideoxyribonucleotides. For RNA, chain-elongating nucleotides include ribonucleotides and chain-terminating nucleotides include 3'-deoxyribonucleotides. The term nucleotide is also well known in the art. For the purposes of this invention,
25 nucleotides include nucleoside mono-, di-, and triphosphates. Nucleotides also include modified nucleotides such as phosphorothioate nucleotides.

Since mass spectrometry is a serial method, in contrast to currently used slab gel electrophoresis which allows several samples to be processed in parallel, in
another embodiment of this invention, a further improvement can be achieved by
30 multiplex mass spectrometric DNA sequencing to allow simultaneous sequencing of more than one DNA or RNA fragment. As described in more detail below, the range of about

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300 mass units between one nucleotide addition can be utilized by employing either mass-modified nucleic acid sequencing primers or chain-elongating and/or terminating nucleoside triphosphates so as to shift the molecular weight of the base-specifically terminated fragments of a particular DNA or RNA species being sequenced in a predetermined manner. For the first time, several sequencing reactions can be mass spectrometrically analyzed in parallel. In yet another embodiment of this invention, multiplex mass spectrometric DNA sequencing can be performed by mass modifying the fragment families through specific oligonucleotides (tag probes) which hybridize to specific tag sequences within each of the fragment families. In another embodiment, the tag probe can be covalently attached to the individual and specific tag sequence prior to mass spectrometry.

Preferred mass spectrometer formats for use in the invention are matrix assisted laser desorption ionization (MALDI), electrospray (ES), ion cyclotron resonance (ICR) and Fourier Transform. For ES, the samples, dissolved in water or in a volatile buffer, are injected either continuously or discontinuously into an atmospheric pressure ionization interface (API) and then mass analyzed by a quadrupole. The generation of multiple ion peaks which can be obtained using ES mass spectrometry can increase the accuracy of the mass determination. Even more detailed information on the specific structure can be obtained using an MS/MS quadrupole configuration.

In MALDI mass spectrometry, various mass analyzers can be used, e.g., magnetic sector/magnetic deflection instruments in single or triple quadrupole mode (MS/MS), Fourier transform and time-of-flight (TOF) configurations as is known in the art of mass spectrometry. For the desorption/ionization process, numerous matrix/laser combinations can be used. Ion-trap and reflectron configurations can also be employed.

In one embodiment of the invention, the molecular weight values of at least two base-specifically terminated fragments are determined concurrently using mass spectrometry. The molecular weight values of preferably at least five and more preferably at least ten base-specifically terminated fragments are determined by mass spectrometry. Also included in the invention are determinations of the molecular weight values of at least 20 base-specifically terminated fragments and at least 30 base-specifically terminated fragments. Further, the nested base-specifically terminated

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fragments in a specific set can be purified of all reactants and by-products but are not separated from one another. The entire set of nested base-specifically terminated fragments is analyzed concurrently and the molecular weight values are determined. At least two base-specifically terminated fragments are analyzed concurrently by mass spectrometry when the fragments are contained in the same sample.

In general, the overall mass spectrometric DNA sequencing process will start with a library of small genomic fragments obtained after first randomly or specifically cutting the genomic DNA into large pieces which then, in several subcloning steps, are reduced in size and inserted into vectors like derivatives of M13 or pUC (e.g., M13mp18 or M13mp19) (see FIGURE 1). In a different approach, the fragments inserted in vectors, such as M13, are obtained via subcloning starting with a cDNA library. In yet another approach, the DNA fragments to be sequenced are generated by the polymerase chain reaction (e.g., Higuchi *et al.*, "A General Method of in vitro Preparation and Mutagenesis of DNA Fragments: Study of Protein and DNA Interactions," *Nucleic Acids Res.*, 16, 7351-67 (1988)). As is known in the art, Sanger sequencing can start from one nucleic acid primer (UP) binding to the plus-strand or from another nucleic acid primer binding to the opposite minus-strand. Thus, either the complementary sequence of both strands of a given unknown DNA sequence can be obtained (providing for reduction of ambiguity in the sequence determination) or the length of the sequence information obtainable from one clone can be extended by generating sequence information from both ends of the unknown vector-inserted DNA fragment.

The nucleic acid primer carries, preferentially at the 5'-end, a linking functionality, L, which can include a spacer of sufficient length and which can interact with a suitable functionality, L', on a solid support to form a reversible linkage such as a photocleavable bond. Since each of the four Sanger sequencing families starts with a nucleic acid primer (L-UP; FIGURE 1) this fragment family can be bound to the solid support by reacting with functional groups, L', on the surface of a solid support and then intensively washed to remove all buffer salts, triphosphates, enzymes, reaction by-products, etc. Furthermore, for mass spectrometric analysis, it can be of importance at this stage to exchange the cation at the phosphate backbone of the DNA fragments in

- 20 -

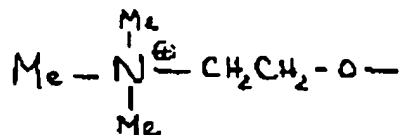
order to eliminate peak broadening due to a heterogeneity in the cations bound per nucleotide unit. Since the L-L' linkage is only of a temporary nature with the purpose to capture the nested Sanger DNA or RNA fragments to properly condition them for mass spectrometric analysis, there are different chemistries which can serve this purpose. In addition to the examples given in which the nested fragments are coupled covalently to the solid support, washed, and cleaved off the support for mass spectrometric analysis, the temporary linkage can be such that it is cleaved under the conditions of mass spectrometry, i.e., a photocleavable bond such as a charge transfer complex or a stable organic radical. Furthermore, the linkage can be formed with L' being a quaternary ammonium group (some examples are given in FIGURE 19). In this case, preferably, the surface of the solid support carries negative charges which repel the negatively charged nucleic acid backbone and thus facilitates desorption. Desorption will take place either by the heat created by the laser pulse and/or, depending on L' by specific absorption of laser energy which is in resonance with the L' chromophore (see, e.g., examples given in FIGURE 19). The functionalities, L and L', can also form a charge transfer complex and thereby form the temporary L-L' linkage. Various examples for appropriate functionalities with either acceptor or donor properties are depicted without limitation in FIGURE 20. Since in many cases the "charge-transfer band" can be determined by UV/vis spectrometry (see e.g. Organic Charge Transfer Complexes by R. Foster, Academic Press, 1969), the laser energy can be tuned to the corresponding energy of the charge-transfer wavelength and, thus, a specific desorption off the solid support can be initiated. Those skilled in the art will recognize that several combinations can serve this purpose and that the donor functionality can be either on the solid support or coupled to the nested Sanger DNA/RNA fragments or vice versa.

In yet another approach, the temporary linkage L-L' can be generated by homolytically forming relatively stable radicals as exemplified in FIGURE 21. In example 4 of FIGURE 21, a combination of the approaches using charge-transfer complexes and stable organic radicals is shown. Here, the nested Sanger DNA/RNA fragments are captured via the formation of a charge transfer complex. Under the influence of the laser pulse, desorption (as discussed above) as well as ionization will take place at the radical position. In the other examples of FIGURE 21 under the influence of the laser pulse, the

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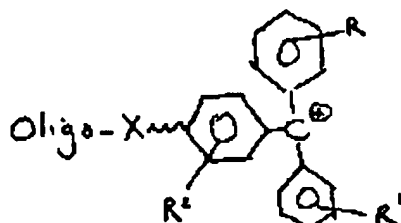
L-L' linkage will be cleaved and the nested Sanger DNA/RNA fragments desorbed and subsequently ionized at the radical position formed. Those skilled in the art will recognize that other organic radicals can be selected and that, in relation to the dissociation energies needed to homolytically cleave the bond between them, a
 5 corresponding laser wavelength can be selected (see e.g. Reactive Molecules by C. Wentrup, John Wiley & Sons, 1984). In yet another approach, the nested Sanger DNA/RNA fragments are captured via Watson-Crick base pairing to a solid support-bound oligonucleotide complementary to either the sequence of the nucleic acid primer or the tag oligonucleotide sequence (see FIGURE 22). The duplex formed will be cleaved
 10 under the influence of the laser pulse and desorption can be initiated. The solid support-bound base sequence can be presented through natural oligoribo- or oligodeoxyribonucleotide as well as analogs (e.g. thio-modified phosphodiester or phosphotriester backbone) or employing oligonucleotide mimetics such as PNA analogs (see e.g. Nielsen *et al.*, Science, 254, 1497 (1991)) which render the base sequence less
 15 susceptible to enzymatic degradation and hence increases overall stability of the solid support-bound capture base sequence. With appropriate bonds, L-L', a cleavage can be obtained directly with a laser tuned to the energy necessary for bond cleavage. Thus, the immobilized nested Sanger fragments can be directly ablated during mass spectrometric analysis.

20 Prior to mass spectrometric analysis, it may be useful to "condition" nucleic acid molecules, for example to decrease the laser energy required for volatilization, to minimize fragmentation or to otherwise increase the sensitivity of mass spectrometric detection. For example, nucleic acids can be "conditioned" by adding positive or negative charges, i.e. charge tags (CTs). CTs increase the mass spectrometer detection
 25 sensitivity by increasing the degree of ionization during the mass spectrometric (e.g. MALDI) process. A CT can be linked either to the external 3' or 5' position or internally e.g. at the 2' position or at the base, e.g. at C-5 in uracil, C-5 methylgroup of thymine, C-5 at cytosine, at C⁷ or C⁸ of guanine, adenine and hypoxanthine or at the phosphate ester moiety. Charge tags, CTs, can function molecules with permanent (i.e.
 30 pH-independent) ionization, such as:



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or molecules which generate a positive charge upon MALDI and which are stabilized by delocalization of the positive charge by mesomeric effects in unsaturated and/or aromatic systems such as:



wherein, $R, R^1, R^2 = H, \text{OAl}$ (wherein Al = e.g. lower alkyl, methyl, ethyl, propyl), NO_2 , CN, CO_2H , CO_2 active ester, or halogen; and
 $X = -\text{O}-, -\text{NH}-, -\text{S}-, \text{C}=\text{O}, \text{OCO}$ either in the para or meta position.

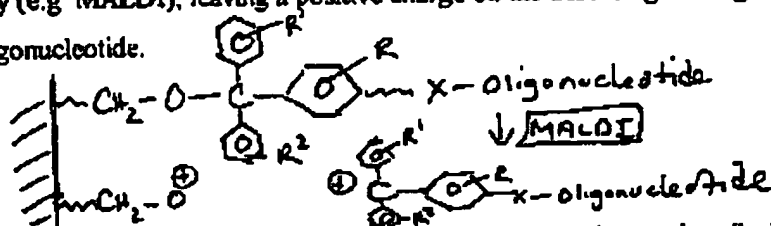
10

For example, the positive charge of a trityl cation is produced during MALDI by the removal of a moiety such as: $-\text{OR}$, where $R =$ a lower alkyl, or an anion such as ClO_4^- , SbF_6^- , BF_4^- and the like.

15

In an alternative scheme, the trityl group is used to anchor the oligonucleotide to a solid support via the tertiary carbon and this bond is cleaved during mass spectrometry (e.g. MALDI), leaving a positive charge on the desorbing and high vacuum flying oligonucleotide.

20



One of skill in the art can readily appreciate several variations to the schemes described above. In addition to employing the charge tag array alone, one of skill in the art can employ a charge tag array in conjunction with another conditioning means. Particularly preferred means to be used in conjunction with the CT include treating the phosphodiester bond with trialkylsilyl halides or the phosphomonothiodiester bond with alkyl iodides to render the polyanionic backbone neutral.

25

30

Another example of conditioning is modification of the phosphodiester backbone of the nucleic acid molecule (e.g. cation exchange), which can be useful for eliminating peak broadening due to a heterogeneity in the cations bound per nucleotide unit. In addition, a nucleic acid molecule can be contacted with an alkylating agent such as alkyl iodide, iodoacetamide, β -iodoethanol, or 2,3-epoxy-1-propanol, the monothio phosphodiester bonds of a nucleic acid molecule can be transformed into a phosphotriester bond. Likewise, phosphodiester bonds may be transformed to uncharged derivatives employing trialkylsilyl chlorides. Further conditioning involves incorporating nucleotides which reduce sensitivity for depurination (fragmentation during MS) such as N7- or N9-deazapurine nucleotides, or RNA building blocks or using oligonucleotide triesters or incorporating phosphorothioate functions which are alkylated or employing oligonucleotide mimetics such as PNA.

Modification of the phosphodiester backbone can be accomplished by, for example, using alpha-thio modified nucleotides for chain elongation and termination. With alkylating agents such as alkyl iodides, iodoacetamide, β -iodoethanol, 2,3-epoxy-1-propanol (see FIGURE 10), the monothio phosphodiester bonds of the nested Sanger fragments are transformed into phosphotriester bonds. Multiplexing by mass modification in this case is obtained by mass-modifying the nucleic acid primer (UP) or the nucleoside triphosphates at the sugar or the base moiety. To those skilled in the art, other modifications of the nested Sanger fragments can be envisioned. In one embodiment of the invention, the linking chemistry allows one to cleave off the so-purified nested DNA enzymatically, chemically or physically. By way of example, the L-L' chemistry can be of a type of disulfide bond (chemically cleavable, for example, by mercaptoethanol or dithioerythrol), a biotin/streptavidin system, a heterobifunctional derivative of a trityl ether group (Köster *et al.*, "A Versatile Acid-Labile Linker for Modification of Synthetic Biomolecules," *Tetrahedron Letters* **31**, 7095 (1990)) which can be cleaved under mildly acidic conditions, a levulinyl group cleavable under almost neutral conditions with a hydrazinium/acetate buffer, an arginine-arginine or lysine-lysine bond cleavable by an endopeptidase enzyme like trypsin or a pyrophosphate bond cleavable by a pyrophosphatase, a photocleavable bond which can be, for example, physically cleaved and the like (see, e.g., FIGURE 23). Optionally, another cation

exchange can be performed prior to mass spectrometric analysis. In the instance that an enzyme-cleavable bond is utilized to immobilize the nested fragments, the enzyme used to cleave the bond can serve as an internal mass standard during MS analysis.

5 The purification process and/or ion exchange process can be carried out by a number of other methods instead of, or in conjunction with, immobilization on a solid support. For example, the base-specifically terminated products can be separated from the reactants by dialysis, filtration (including ultrafiltration), and chromatography. Likewise, these techniques can be used to exchange the cation of the phosphate backbone with a counter-ion which reduces peak broadening.

10 The base-specifically terminated fragment families can be generated by standard Sanger sequencing using the Large Klenow fragment of *E. coli* DNA polymerase I, by Sequenase, Taq DNA polymerase and other DNA polymerases suitable for this purpose, thus generating nested DNA fragments for the mass spectrometric analysis. It is, however, part of this invention that base-specifically terminated RNA
15 transcripts of the DNA fragments to be sequenced can also be utilized for mass spectrometric sequence determination. In this case, various RNA polymerases such as the SP6 or the T7 RNA polymerase can be used on appropriate vectors containing, for example, the SP6 or the T7 promoters (e.g. Axelrod *et al.*, "Transcription from Bacteriophage T7 and SP6 RNA Polymerase Promoters in the Presence of 3'-
20 Deoxyribonucleoside 5'-triphosphate Chain Terminators," *Biochemistry* 24, 5716-23 (1985)). In this case, the unknown DNA sequence fragments are inserted downstream from such promoters. Transcription can also be initiated by a nucleic acid primer (Pitulle *et al.*, "Initiator Oligonucleotides for the Combination of Chemical and Enzymatic RNA
25 Synthesis," *Gene* 112, 101-105 (1992)) which carries, as one embodiment of this invention, appropriate linking functionalities, L, which allow the immobilization of the nested RNA fragments, as outlined above, prior to mass spectrometric analysis for purification and/or appropriate modification and/or conditioning.

For this immobilization process of the DNA/RNA sequencing products for mass spectrometric analysis, various solid supports can be used. e.g., beads (silica gel,
30 controlled pore glass, magnetic beads, Sephadex/Sepharose beads, cellulose beads, etc.), capillaries, glass fiber filters, glass surfaces, metal surfaces or plastic material. Examples

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of useful plastic materials include membranes in filter or microtiter plate formats, the latter allowing the automation of the purification process by employing microtiter plates which, as one embodiment of the invention, carry a permeable membrane in the bottom of the well functionalized with L'. Membranes can be based on polyethylene, polypropylene, polyamide, polyvinylidenedifluoride and the like. Examples of suitable metal surfaces include steel, gold, silver, aluminum, and copper. After purification, cation exchange, and/or modification of the phosphodiester backbone of the L-L' bound nested Sanger fragments, they can be cleaved off the solid support chemically, enzymatically or physically. Also, the L-L' bound fragments can be cleaved from the support when they are subjected to mass spectrometric analysis by using appropriately chosen L-L' linkages and corresponding laser energies/intensities as described above and in FIGURES 19-23.

The highly purified, four base-specifically terminated DNA or RNA fragment families are then analyzed with regard to their fragment lengths via determination of their respective molecular weights by MALDI or ES mass spectrometry.

For ES, the samples, dissolved in water or in a volatile buffer, are injected either continuously or discontinuously into an atmospheric pressure ionization interface (API) and then mass analyzed by a quadrupole. With the aid of a computer program, the molecular weight peaks are searched for the known molecular weight of the nucleic acid primer (UP) and determined which of the four chain-terminating nucleotides has been added to the UP. This represents the first nucleotide of the unknown sequence. Then, the second, the third, the n^{th} extension product can be identified in a similar manner and, by this, the nucleotide sequence is assigned. The generation of multiple ion peaks which can be obtained using ES mass spectrometry can increase the accuracy of the mass determination.

In MALDI mass spectrometry, various mass analyzers can be used, e.g., magnetic sector/magnetic deflection instruments in single or triple quadrupole mode (MS/MS), Fourier transform and time-of-flight (TOF) configurations as is known in the art of mass spectrometry. FIGURES 2A through 6 are given as an example of the data obtainable when sequencing a hypothetical DNA fragment of 50 nucleotides in length (SEQ ID NO:3) and having a molecular weight of 15,344.02 daltons. The molecular weights calculated for the ddT (FIGURES 2A and 2B), ddA (FIGURES 3A and 3B),

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ddG (FIGURES 4A and 4B) and ddC (FIGURES 5A and 5B) terminated products are given (corresponding to fragments of SEQ ID NO:3) and the idealized four MALDI-TOF mass spectra shown. All four spectra are superimposed, and from this, the DNA sequence can be generated. This is shown in the summarizing FIGURE 6, demonstrating how the molecular weights are correlated with the DNA sequence. MALDI-TOF spectra have been generated for the ddT terminated products (FIGURE 16) corresponding to those shown in FIGURE 2 and these spectra have been superimposed (FIGURE 17). The correlation of calculated molecular weights of the ddT fragments and their experimentally-verified weights are shown in Table 1. Likewise, if all four chain-terminating reactions are combined and then analyzed by mass spectrometry, the molecular weight difference between two adjacent peaks can be used to determine the sequence. For the desorption/ionization process, numerous matrix/laser combinations can be used.

TABLE I

Correlation of calculated and experimentally verified molecular weights of the 13 DNA fragments of FIGURES 2 and 16.

Fragment (n-mer)	calculated mass	experimental mass	difference
7-mer	2104.45	2119.9	+15.4
10-mer	3011.04	3026.1	+15.1
11-mer	3315.24	3330.1	+14.9
19-mer	5771.82	5788.0	+16.2
20-mer	6076.02	6093.8	+17.8
24-mer	7311.82	7374.9	+63.1
26-mer	7945.22	7960.9	+15.7
33-mer	10112.63	10125.3	+12.7
37-mer	11348.43	11361.4	+13.0
38-mer	11652.62	11670.2	+17.6
42-mer	12872.42	12888.3	+15.9
46-mer	14108.22	14125.0	+16.8
50-mer	15344.02	15362.6	+18.6

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In order to increase throughput to a level necessary for high volume genomic and cDNA sequencing projects, a further embodiment of the present invention is to utilize multiplex mass spectrometry to simultaneously determine more than one sequence. This can be achieved by several, albeit different, methodologies, the basic principle being the mass modification of the nucleic acid primer (UP), the chain-elongating and/or terminating nucleoside triphosphates, or by using mass-differentiated tag probes hybridizable to specific tag sequences. The term "nucleic acid primer" as used herein encompasses primers for both DNA and RNA Sanger sequencing.

By way of example, FIGURE 7 presents a general formula of the nucleic acid primer (UP) and the tag probes (TP). The mass modifying moiety can be attached, for instance, to either the 5'-end of the oligonucleotide (M^1), to the nucleobase (or bases) (M^2 , M^7), to the phosphate backbone (M^3), and to the 2'-position of the nucleoside (nucleosides) (M^4 , M^6) or/and to the terminal 3'-position (M^5). Primer length can vary between 1 and 50 nucleotides in length. For the priming of DNA Sanger sequencing, the primer is preferentially in the range of about 15 to 30 nucleotides in length. For artificially priming the transcription in a RNA polymerase-mediated Sanger sequencing reaction, the length of the primer is preferentially in the range of about 2 to 6 nucleotides. If a tag probe (TP) is to hybridize to the integrated tag sequence of a family chain-terminated fragments, its preferential length is about 20 nucleotides.

The table in FIGURE 7 depicts some examples of mass-modified primer/tag probe configurations for DNA, as well as RNA, Sanger sequencing. This list is, however, not meant to be limiting, since numerous other combinations of mass-modifying functions and positions within the oligonucleotide molecule are possible and are deemed part of the invention. The mass-modifying functionality can be, for example, a halogen, an azido, or of the type, XR, wherein X is a linking group and R is a mass-modifying functionality. The mass-modifying functionality can thus be used to introduce defined mass increments into the oligonucleotide molecule.

In another embodiment, the nucleotides used for chain-elongation and/or termination are mass-modified. Examples of such modified nucleotides are shown in FIGURE 8. Here the mass-modifying moiety, M, can be attached either to the nucleobase, M^2 (in case of the c^7 -deazanucleosides also to C-7, M^7), to the triphosphate

group at the alpha phosphate, M^3 , or to the 2'-position of the sugar ring of the nucleoside triphosphate, M^4 and M^6 . Furthermore, the mass-modifying functionality can be added so as to affect chain termination, such as by attaching it to the 3'-position of the sugar ring in the nucleoside triphosphate, M^5 . The list in FIGURE 8 represents examples of possible configurations for generating chain-terminating nucleoside triphosphates for RNA or DNA Sanger sequencing. For those skilled in the art, however, it is clear that many other combinations can serve the purpose of the invention equally well. In the same way, those skilled in the art will recognize that chain-elongating nucleoside triphosphates can also be mass-modified in a similar fashion with numerous variations and combinations in functionality and attachment positions.

Without limiting the scope of the invention, FIGURE 9 gives a more detailed description of particular examples of how the mass-modification, M , can be introduced for X in XR as well as using oligo-/polyethylene glycol derivatives for R . The mass-modifying increment in this case is 44, i.e. five different mass-modified species can be generated by just changing m from 0 to 4 thus adding mass units of 45 ($m=0$), 89 ($m=1$), 133 ($m=2$), 177 ($m=3$) and 221 ($m=4$) to the nucleic acid primer (UP), the tag probe (TP) or the nucleoside triphosphates respectively. The oligo/polyethylene glycols can also be monoalkylated by a lower alkyl such as methyl, ethyl, propyl, isopropyl, *t*-butyl and the like. A selection of linking functionalities, X , are also illustrated. Other chemistries can be used in the mass-modified compounds, as for example, those described recently in Oligonucleotides and Analogues, A Practical Approach, F. Eckstein, editor, IRL Press, Oxford, 1991.

In yet another embodiment, various mass-modifying functionalities, R , other than oligo/polyethylene glycols, can be selected and attached via appropriate linking chemistries, X . Without any limitation, some examples are given in FIGURE 10. A simple mass-modification can be achieved by substituting H for halogens like F, Cl, Br and/or I, or pseudohalogens such as SCN, NCS, or by using different alkyl, aryl or aralkyl moieties such as methyl, ethyl, propyl, isopropyl, *t*-butyl, hexyl, phenyl, substituted phenyl, benzyl, or functional groups such as CH_2F , CHF_2 , CF_3 , $Si(CH_3)_3$, $Si(CH_3)_2(C_2H_5)$, $Si(CH_3)(C_2H_5)_2$, $Si(C_2H_5)_3$. Yet another mass-modification can be obtained by attaching homo- or heteropeptides through X to the UP, TP or nucleoside

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triphosphates. One example useful in generating mass-modified species with a mass increment of 57 is the attachment of oligoglycines, e.g., mass-modifications of 74 ($r=1$, $m=0$), 131 ($r=1$, $m=2$), 188 ($r=1$, $m=3$), 245 ($r=1$, $m=4$) are achieved. Simple oligoamides also can be used, e.g., mass-modifications of 74 ($r=1$, $m=0$), 88 ($r=2$, $m=0$), 102 ($r=3$, $m=0$), 116 ($r=4$, $m=0$), etc. are obtainable. For those skilled in the art, it will be obvious that there are numerous possibilities in addition to those given in FIGURE 10 and the above mentioned reference (Oligonucleotides and Analogues, F. Eckstein, 1991), for introducing, in a predetermined manner, many different mass-modifying functionalities to UP, TP and nucleoside triphosphates which are acceptable for DNA and RNA Sanger sequencing.

As used herein, the superscript 0-i designates $i + 1$ mass differentiated nucleotides, primers or tags. In some instances, the superscript 0 (e.g., NTP^0 , UP^0) can designate an unmodified species of a particular reactant, and the superscript i (e.g., NTP^i , NTP^1 , NTP^2 , etc.) can designate the i -th mass-modified species of that reactant. If, for example, more than one species of nucleic acids (e.g., DNA clones) are to be concurrently sequenced by multiplex DNA sequencing, then $i + 1$ different mass-modified nucleic acid primers (UP^0 , UP^1 , ..., UP^i) can be used to distinguish each set of base-specifically terminated fragments, wherein each species of mass-modified UP^i can be distinguished by mass spectrometry from the rest.

As illustrative embodiments of this invention, three different basic processes for multiplex mass spectrometric DNA sequencing employing the described mass-modified reagents are described below:

- A) Multiplexing by the use of mass-modified nucleic acid primers (UP) for Sanger DNA or RNA sequencing (see for example FIGURE 11);
- B) Multiplexing by the use of mass-modified nucleoside triphosphates as chain elongators and/or chain terminators for Sanger DNA or RNA sequencing (see for example FIGURE 12); and
- C) Multiplexing by the use of tag probes which specifically hybridize to tag sequences which are integrated into part of the four Sanger DNA/RNA base-specifically terminated fragment families. Mass modification here can be achieved as described for FIGURES 7, 9 and 10,

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or alternately, by designing different oligonucleotide sequences having the same or different length with unmodified nucleotides which, in a predetermined way, generate appropriately differentiated molecular weights (see for example FIGURE 13).

5 The process of multiplexing by mass-modified nucleic acid primers (UP) is illustrated by way of example in FIGURE 11 for mass analyzing four different DNA clones simultaneously. The first reaction mixture is obtained by standard Sanger DNA sequencing having unknown DNA fragment 1 (clone 1) integrated in an appropriate vector (e.g., M13mp18), employing an unmodified nucleic acid primer UP^0 , and a standard mixture of the four unmodified deoxynucleoside triphosphates, $dNTP^0$, and with 1/10th of one of the four dideoxynucleoside triphosphates, $ddNTP^0$. A second reaction mixture for DNA fragment 2 (clone 2) is obtained by employing a mass-modified nucleic acid primer UP^1 and, as before, the four unmodified nucleoside triphosphates, $dNTP^0$, containing in each separate Sanger reaction 1/10th of the chain-terminating unmodified dideoxynucleoside triphosphates $ddNTP^0$. In the other two experiments, the four Sanger reactions have the following compositions: DNA fragment 3 (clone 3), UP^2 , $dNTP^0$, $ddNTP^0$ and DNA fragment 4 (clone 4), UP^3 , $dNTP^0$, $ddNTP^0$. For mass spectrometric DNA sequencing, all base-specifically terminated reactions of the four clones are pooled and mass analyzed. The various mass peaks belonging to the four dideoxy-terminated (e.g., ddT-terminated) fragment families are assigned to specifically elongated and ddT-terminated fragments by searching (such as by a computer program) for the known molecular ion peaks of UP^0 , UP^1 , UP^2 and UP^3 extended by either one of the four dideoxynucleoside triphosphates, UP^0 - ddN^0 , UP^1 - ddN^0 , UP^2 - ddN^0 and UP^3 - ddN^0 . In this way, the first nucleotides of the four unknown DNA sequences of clone 1 to 4 are determined. The process is repeated, having memorized the molecular masses of the four specific first extension products, until the four sequences are assigned. Unambiguous mass/sequence assignments are possible even in the worst case scenario in which the four mass-modified nucleic acid primers are extended by the same dideoxynucleoside triphosphate, the extension products then being, for example, UP^0 -ddT, UP^1 -ddT, UP^2 -ddT and UP^3 -ddT, which differ by the known mass increment differentiating the four nucleic acid primers. In another embodiment of this invention, an

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analogous technique is employed using different vectors containing, for example, the SP6 and/or T7 promoter sequences, and performing transcription with the nucleic acid primers UP^0 , UP^1 , UP^2 and UP^3 and either an RNA polymerase (e.g., SP6 or T7 RNA polymerase) with chain-elongating and terminating unmodified nucleoside triphosphates NTP^0 and $3'-dNTP^0$. Here, the DNA sequence is being determined by Sanger RNA sequencing.

FIGURE 12 illustrates the process of multiplexing by mass-modified chain-elongating or/and terminating nucleoside triphosphates in which three different DNA fragments (3 clones) are mass analyzed simultaneously. The first DNA Sanger sequencing reaction (DNA fragment 1, clone 1) is the standard mixture employing unmodified nucleic acid primer UP^0 , $dNTP^0$ and in each of the four reactions one of the four $ddNTP^0$. The second (DNA fragment 2, clone 2) and the third (DNA fragment 3, clone 3) have the following contents: UP^0 , $dNTP^0$, $ddNTP^1$ and UP^0 , $dNTP^0$, $ddNTP^2$, respectively. In a variation of this process, an amplification of the mass increment in mass-modifying the extended DNA fragments can be achieved by either using an equally mass-modified deoxynucleoside triphosphate (i.e., $dNTP^1$, $dNTP^2$) for chain elongation alone or in conjunction with the homologous equally mass-modified dideoxynucleoside triphosphate. For the three clones depicted above, the contents of the reaction mixtures can be as follows: either $UP^0/dNTP^0/ddNTP^0$, $UP^0/dNTP^1/ddNTP^0$ and $UP^0/dNTP^2/ddNTP^0$ or $UP^0/dNTP^0/ddNTP^0$, $UP^0/dNTP^1/ddNTP^1$ and $UP^0/dNTP^2/ddNTP^2$. As described above, DNA sequencing can be performed by Sanger RNA sequencing employing unmodified nucleic acid primers, UP^0 , and an appropriate mixture of chain-elongating and terminating nucleoside triphosphates. The mass-modification can be again either in the chain-terminating nucleoside triphosphate alone or in conjunction with mass-modified chain-elongating nucleoside triphosphates. Multiplexing is achieved by pooling the three base-specifically terminated sequencing reactions (e.g., the $ddTTP$ terminated products) and simultaneously analyzing the pooled products by mass spectrometry. Again, the first extension products of the known nucleic acid primer sequence are assigned, e.g., via a computer program. Mass/sequence assignments are possible even in the worst case in which the nucleic acid primer is extended/terminated by the same nucleotide, e.g., ddT , in all three clones. The following

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configurations thus obtained can be well differentiated by their different mass-modifications: UP^0-ddT^0 , UP^0-ddT^1 , UP^0-ddT^2 .

In yet another embodiment of this invention, DNA sequencing by multiplex mass spectrometry can be achieved by cloning the DNA fragments to be sequenced in "plex-vectors" containing vector specific "tag sequences" as described (Köster *et al.*, "Oligonucleotide Synthesis and Multiplex DNA Sequencing Using Chemiluminescent Detection," *Nucleic Acids Res. Symposium Ser. No. 24*, 318-321 (1991)); then pooling clones from different plex-vectors for DNA preparation and the four separate Sanger sequencing reactions using standard $dNTP^0/ddNTP^0$ and nucleic acid primer UP^0 ,
10 purifying the four multiplex fragment families via linking to a solid support through the linking group, L, at the 5'-end of UP; washing out all by-products, and cleaving the purified multiplex DNA fragments off the support or using the L-L' bound nested Sanger fragments as such for mass spectrometric analysis as described above; performing demultiplexing by one-by-one hybridization of specific "tag probes"; and subsequently
15 analyzing by mass spectrometry (see, for example, FIGURE 13). As a reference point, the four base-specifically terminated multiplex DNA fragment families are run by the mass spectrometer and all ddT^0 -, ddA^0 -, ddC^0 - and ddG^0 -terminated molecular ion peaks are respectively detected and memorized. Assignment of, for example, ddT^0 -terminated DNA fragments to a specific fragment family is accomplished by another mass
20 spectrometric analysis after hybridization of the specific tag probe (TP) to the corresponding tag sequence contained in the sequence of this specific fragment family. Only those molecular ion peaks which are capable of hybridizing to the specific tag probe are shifted to a higher molecular mass by the same known mass increment (e.g. of the tag probe). These shifted ion peaks, by virtue of all hybridizing to a specific tag probe,
25 belong to the same fragment family. For a given fragment family, this is repeated for the remaining chain terminated fragment families with the same tag probe to assign the complete DNA sequence. This process is repeated $i-1$ times corresponding to i clones multiplexed (the
30 i -th clone is identified by default).

The differentiation of the tag probes for the different multiplexed clones can be obtained just by the DNA sequence and its ability to Watson-Crick base pair to the tag

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sequence. It is well known in the art how to calculate stringency conditions to provide for specific hybridization of a given tag probe with a given tag sequence (see, for example, Molecular Cloning. A laboratory manual 2ed, ed. by Sambrook, Fritsch and Maniatis (Cold Spring Harbor Laboratory Press: NY, 1989, Chapter 11). Furthermore, differentiation can be obtained by designing the tag sequence for each plex-vector to have a sufficient mass difference so as to be unique just by changing the length or base composition or by mass-modifications according to FIGURES 7, 9 and 10. In order to keep the duplex between the tag sequence and the tag probe intact during mass spectrometric analysis, it is another embodiment of the invention to provide for a covalent attachment mediated by, for example, photoreactive groups such as psoralen and ellipticine and by other methods known to those skilled in the art (see, for example, Hélène *et al.*, Nature **344**, 358 (1990) and Thuong *et al.* "Oligonucleotides Attached to Intercalators, Photoreactive and Cleavage Agents" in F. Eckstein, Oligonucleotides and Analogues: A Practical Approach, IRL Press, Oxford 1991, 283-306).

The DNA sequence is unraveled again by searching for the lowest molecular weight molecular ion peak corresponding to the known UP^0 -tag sequence/tag probe molecular weight plus the first extension product, e.g., ddT^0 , then the second, the third, etc.

In a combination of the latter approach with the previously described multiplexing processes, a further increase in multiplexing can be achieved by using, in addition to the tag probe/tag sequence interaction, mass-modified nucleic acid primers (FIGURE 7) and/or mass-modified deoxynucleoside, $dNTP^{0-i}$, and/or dideoxynucleoside triphosphates, $ddNTP^{0-i}$. Those skilled in the art will realize that the tag sequence/tag probe multiplexing approach is not limited to Sanger DNA sequencing generating nested DNA fragments with DNA polymerases. The DNA sequence can also be determined by transcribing the unknown DNA sequence from appropriate promoter-containing vectors (see above) with various RNA polymerases and mixtures of $NTP^{0-i}/3'-dNTP^{0-i}$, thus generating nested RNA fragments.

In yet another embodiment of this invention, the mass-modifying functionality can be introduced by a two or multiple step process. In this case, the nucleic acid primer, the chain-elongating or terminating nucleoside triphosphates and/or

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the tag probes are, in a first step, modified by a precursor functionality such as azido, -N₃, or modified with a functional group in which the R in XR is H (FIGURE 7, 9) thus providing temporary functions, e.g., but not limited to -OH, -NH₂, -NHR, -SH, -NCS, -OCO(CH₂)_rCOOH (r = 1-20), -NHCO(CH₂)_rCOOH (r = 1-20), -OSO₂OH, -OCO(CH₂)_rI (r = 1-20), -OP(O-Alkyl)N(Alkyl)₂. These less bulky functionalities result in better substrate properties for the enzymatic DNA or RNA synthesis reactions of the DNA sequencing process. The appropriate mass-modifying functionality is then introduced after the generation of the nested base-specifically terminated DNA or RNA fragments prior to mass spectrometry. Several examples of compounds which can serve as mass-modifying functionalities are depicted in FIGURES 9 and 10 without limiting the scope of this invention.

Another aspect of this invention concerns kits for sequencing nucleic acids by mass spectrometry which include combinations of the above-described sequencing reactants. For instance, in one embodiment, the kit comprises reactants for multiplex mass spectrometric sequencing of several different species of nucleic acid. The kit can include a solid support having a linking functionality (L¹) for immobilization of the base-specifically terminated products; at least one nucleic acid primer having a linking group (L) for reversibly and temporarily linking the primer and solid support through, for example, a photocleavable bond; a set of chain-elongating nucleotides (e.g., dATP, dCTP, dGTP and dTTP, or ATP, CTP, GTP and UTP); a set of chain-terminating nucleotides (such as 2',3'-dideoxynucleotides for DNA synthesis or 3'-deoxynucleotides for RNA synthesis); and an appropriate polymerase for synthesizing complementary nucleotides. Primers and/or terminating nucleotides can be mass-modified so that the base-specifically terminated fragments generated from one of the species of nucleic acids to be sequenced can be distinguished by mass spectrometry from all of the others. Alternative to the use of mass-modified synthesis reactants, a set of tag probes (as described above) can be included in the kit. The kit can also include appropriate buffers as well as instructions for performing multiplex mass spectrometry to concurrently sequence multiple species of nucleic acids.

In another embodiment, a nucleic acid sequencing kit can comprise a solid support as described above, a primer for initiating synthesis of complementary nucleic

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acid fragments, a set of chain-elongating nucleotides and an appropriate polymerase. The mass-modified chain-terminating nucleotides are selected so that the addition of one of the chain terminators to a growing complementary nucleic acid can be distinguished by mass spectrometry.

5 The present invention is further illustrated by the following examples which should not be construed as limiting in any way. The contents of all cited references (including literature references, issued patents, published patent applications (including international patent application Publication Number WO 94/16101, entitled "DNA Sequencing by Mass Spectrometry" by H. Koester; and international patent application
10 Publication Number WO 94/21822 entitled "DNA Sequencing by Mass Spectrometry Via Exonuclease Degradation" by H. Koester), and co-pending patent applications, (including U.S Patent Application Serial No. 08/406,199, entitled "DNA Diagnostics Based on Mass Spectrometry" by H. Koester), as cited throughout this application are hereby expressly incorporated by reference.

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EXAMPLE 1

Immobilization of primer-extension products of Sanger DNA sequencing reaction for mass spectrometric analysis via disulfide bonds.

20 As a solid support, Sequelon membranes (Millipore Corp., Bedford, MA) with phenyl isothiocyanate groups are used as a starting material. The membrane disks, with a diameter of 8 mm, are wetted with a solution of N-methylmorpholine/water/2-propanol (NMM solution) (2/49/49 v/v/v), the excess liquid removed with filter paper and placed on a piece of plastic film or aluminum foil located on a heating block set to
25 55°C. A solution of 1 mM 2-mercaptoethylamine (cysteamine) or 2, 2'-dithio-bis(ethylamine) (cystamine) or S-(2-thiopyridyl)-2-thio-ethylamine (10 ul, 10 nmol) in NMM is added per disk and heated at 55°C. After 15 min, 10 ul of NMM solution are added per disk and heated for another 5 min. Excess of isothiocyanate groups may be removed by treatment with 10 ul of a 10 mM solution of glycine in NMM solution. For
30 cystamine, the disks are treated with 10 ul of a solution of 1M aqueous dithiothreitol (DTT)/2-propanol (1:1 v/v) for 15 min at room temperature. Then, the disks are

thoroughly washed in a filtration manifold with 5 aliquots of 1 ml each of the NMM solution, then with 5 aliquots of 1 ml acetonitrile/water (1/1 v/v) and subsequently dried. If not used immediately the disks are stored with free thiol groups in a solution of 1M aqueous dithiothreitol/2-propanol (1:1 v/v) and, before use, DTT is removed by three washings with 1 ml each of the NMM solution. The primer oligonucleotides with 5'-SH functionality can be prepared by various methods (e.g., B.C.F Chu *et al.*, Nucleic Acids Res. **14**, 5591-5603 (1986), Sproat *et al.*, Nucleic Acids Res. **15**, 4837-48 (1987) and Oligonucleotides and Analogues: A Practical Approach (F. Eckstein, editor), IRL Press Oxford, 1991). Sequencing reactions according to the Sanger protocol are performed in a standard way (e.g., H. Swerdlow *et al.*, Nucleic Acids Res. **18**, 1415-19 (1990)). In the presence of about 7-10 mM DTT the free 5'-thiol primer can be used, in other cases, the SH functionality can be protected, e.g., by a trityl group during the Sanger sequencing reactions and removed prior to anchoring to the support in the following way. The four sequencing reactions (150 ul each in an Eppendorf tube) are terminated by a 10 min incubation at 70°C to denature the DNA polymerase (such as Klenow fragment, Sequenase) and the reaction mixtures are ethanol precipitated. The supernatants are removed and the pellets vortexed with 25 ul of an 1M aqueous silver nitrate solution, and after one hour at room temperature, 50 ul of an 1 M aqueous solution of DTT is added and mixed by vortexing. After 15 min, the mixtures are centrifuged and the pellets are washed twice with 100 ul ethylacetate by vortexing and centrifugation to remove excess DTT. The primer extension products with free 5'-thiol group are now coupled to the thiolated membrane supports under mild oxidizing conditions. In general, it is sufficient to add the 5'-thiolated primer extension products dissolved in 10 ul 10 mM de-aerated triethylammonium acetate buffer (TEAA) pH 7.2 to the thiolated membrane supports. Coupling is achieved by drying the samples onto the membrane disks with a cold fan. This process can be repeated by wetting the membrane with 10 ul of 10 mM TEAA buffer pH 7.2 and drying as before. When using the 2-thiopyridyl derivatized compounds, anchoring can be monitored by the release of pyridine-2-thione spectrophotometrically at 343 nm

In another variation of this approach, the oligonucleotide primer is functionalized with an amino group at the 5'-end which is introduced by standard

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procedures during automated DNA synthesis. After primer extension, during the Sanger sequencing process, the primary amino group is reacted with 3-(2-pyridyldithio) propionic acid N-hydroxysuccinimide ester (SPDP) and subsequently coupled to the thiolated supports and monitored by the release of pyridyl-2-thione as described above.

5 After denaturation of DNA polymerase and ethanol precipitation of the sequencing products, the supernatants are removed and the pellets dissolved in 10 μ l 10 mM TEAA buffer pH 7.2 and 10 μ l of a 2 mM solution of SPDP in 10 mM TEAA are added. The reaction mixture is vortexed and incubated for 30 min at 25°C. Excess SPDP is then removed by three extractions (vortexing, centrifugation) with 50 μ l each of ethanol and

10 the resulting pellets are dissolved in 10 μ l 10 mM TEAA buffer pH 7.2 and coupled to the thiolated supports (see above).

The primer-extension products are purified by washing the membrane disks three times each with 100 μ l NMM solution and three times with 100 μ l each of 10 mM TEAA buffer pH 7.2. The purified primer-extension products are released by three

15 successive treatments with 10 μ l of 10 mM 2-mercaptoethanol in 10 mM TEAA buffer pH 7.2, lyophilized and analyzed by either ES or MALDI mass spectrometry.

This procedure can also be used for the mass-modified nucleic acid primers UP^{0-i} in an analogous and appropriate way, taking into account the chemical properties of the mass-modifying functionalities.

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EXAMPLE 2

Immobilization of primer-extension products of Sanger DNA sequencing reaction for mass spectrometric analysis via the levulinyl group

25 5-Aminolevulinic acid is protected at the primary amino group with the Fmoc group using 9-fluorenylmethyl N-succinimidyl carbonate and is then transformed into the N-hydroxysuccinimide ester (NHS ester) using N-hydroxysuccinimide and dicyclohexyl carbodiimide under standard conditions. For the Sanger sequencing reactions, nucleic acid primers, UP^{0-i} , are used which are functionalized with a primary

30 amino group at the 5'-end introduced by standard procedures during automated DNA synthesis with aminolinker phosphoramidites as the final synthetic step. Sanger

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sequencing is performed under standard conditions (see above). The four reaction mixtures (150 μ l each in an Eppendorf tube) are heated to 70°C for 10 min to inactivate the DNA polymerase, ethanol precipitated, centrifuged and resuspended in 10 μ l of 10 mM TEAA buffer pH 7.2. 10 μ l of a 2 mM solution of the Fmoc-5-aminolevuliny-NHS ester in 10 mM TEAA buffer is added, vortexed and incubated at 25°C for 30 min. The excess of the reagent is removed by ethanol precipitation and centrifugation. The Fmoc group is cleaved off by resuspending the pellets in 10 μ l of a solution of 20% piperidine in N,N-dimethylformamide/water (1:1 v/v). After 15 min at 25°C, piperidine is thoroughly removed by three precipitations/centrifugations with 100 μ l each of ethanol, the pellets are resuspended in 10 μ l of a solution of N-methylmorpholine, 2-propanol and water (2/10/88 v/v/v) and are coupled to the solid support carrying an isothiocyanate group. In the case of the DITC-Sequelon membrane (Millipore Corp., Bedford, MA), the membranes are prepared as described in EXAMPLE 1 and coupling is achieved on a heating block at 55°C as described above. RNA extension products are immobilized in an analogous way. The procedure can be applied to other solid supports with isothiocyanate groups in a similar manner.

The immobilized primer-extension products are extensively washed three times with 100 μ l each of NMM solution and three times with 100 μ l 10 mM TEAA buffer pH 7.2. The purified primer-extension products are released by three successive treatments with 10 μ l of 100 mM hydrazinium acetate buffer pH 6.5, lyophilized and analyzed by either ES or MALDI mass spectrometry.

EXAMPLE 3

25 Immobilization of primer-extension products of Sanger DNA sequencing reaction for mass spectrometric analysis via a trypsin sensitive linkage

Sequelon DITC membrane disks of 8 mm diameter (Millipore Corp., Bedford, MA) are wetted with 10 μ l of NMM solution (N-methylmorpholine/propanol-2/water, 2/49/49 v/v/v) and a linker arm introduced by reaction with 10 μ l of a 10 mM solution of 1,6-diaminohexane in NMM. The excess diamine is removed by three washing steps with 100 μ l of NMM solution. Using standard peptide synthesis protocols,

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two L-lysine residues are attached by two successive condensations with N-Fmoc-N-tBoc-L-lysine pentafluorophenylester, the terminal Fmoc group is removed with piperidine in NMM and the free α -amino group coupled to 1,4-phenylene diisothiocyanate (DITC). Excess DITC is removed by three washing steps with 100 μ l 2-propanol and the N-tBoc groups removed with trifluoroacetic acid according to standard peptide synthesis procedures. The nucleic acid primer-extension products are prepared from oligonucleotides which carry a primary amino group at the 5'-terminus. The four Sanger DNA sequencing reaction mixtures (150 μ l each in Eppendorf tubes) are heated for 10 min at 70^oC to inactivate the DNA polymerase, ethanol precipitated, and the pellets resuspended in 10 μ l of a solution of N-methylmorpholine, 2-propanol and water (2/10/88 v/v/v). This solution is transferred to the Lys-Lys-DITC membrane disks and coupled on a heating block set at 55^oC. After drying, 10 μ l of NMM solution is added and the drying process repeated.

The immobilized primer-extension products are extensively washed three times with 100 μ l each of NMM solution and three times with 100 μ l each of 10 mM TEAA buffer pH 7.2. For mass spectrometric analysis, the bond between the primer-extension products and the solid support is cleaved by treatment with trypsin under standard conditions and the released products analyzed by either ES or MALDI mass spectrometry with trypsin serving as an internal mass standard.

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EXAMPLE 4

Immobilization of primer-extension products of Sanger DNA sequencing reaction for mass spectrometric analysis via pyrophosphate linkage

The DITC Sequelon membrane (disks of 8 mm diameter) are prepared as described in EXAMPLE 3 and 10 μ l of a 10 mM solution of 3-aminopyridine adenine dinucleotide (APAD) (Sigma) in NMM solution added. The excess APAD is removed by a 10 μ l wash of NMM solution and the disks are treated with 10 μ l of 10 mM sodium periodate in NMM solution (15 min, 25^oC). Excess periodate is removed and the primer-extension products of the four Sanger DNA sequencing reactions (150 μ l each in Eppendorf tubes) employing nucleic acid primers with a primary amino group at the 5'-

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end are ethanol precipitated, dissolved in 10 ul of a solution of N-methylmorpholine/2-propanol/water (2/10/88 v/v/v) and coupled to the 2' 3'-dialdehyde groups of the immobilized NAD analog.

The primer-extension products are extensively washed with the NMM solution (3 times with 100 ul each) and 10 mM TEAA buffer pH 7.2 (3 times with 100 ul each) and the purified primer-extension products are released by treatment with either NADase or pyrophosphatase in 10 mM TEAA buffer at pH 7.2 at 37°C for 15 min, lyophilized and analyzed by either ES or MALDI mass spectrometry, the enzymes serving as internal mass standards.

EXAMPLE 5

Synthesis of nucleic acid primers mass-modified by glycine residues at the 5'-position of the sugar moiety of the terminal nucleoside

Oligonucleotides are synthesized by standard automated DNA synthesis using β -cyanoethylphosphoramidites (H. Köster *et al.*, Nucleic Acids Res. 12, 4539 (1984)) and a 5'-amino group is introduced at the end of solid phase DNA synthesis (e.g. Agrawal *et al.*, Nucleic Acids Res. 14, 6227-45 (1986) or Sproat *et al.*, Nucleic Acids Res. 15, 6181-96 (1987)). The total amount of an oligonucleotide synthesis, starting with 0.25 μ mol CPG-bound nucleoside, is deprotected with concentrated aqueous ammonia, purified via OligoPAKTM Cartridges (Millipore Corp., Bedford, MA) and lyophilized. This material with a 5'-terminal amino group is dissolved in 100 ul absolute N,N-dimethylformamide (DMF) and condensed with 10 μ mole N-Fmoc-glycine pentafluorophenyl ester for 60 min at 25°C. After ethanol precipitation and centrifugation, the Fmoc group is cleaved off by a 10 min treatment with 100 ul of a solution of 20% piperidine in N,N-dimethylformamide. Excess piperidine, DMF and the cleavage product from the Fmoc group are removed by ethanol precipitation and the precipitate lyophilized from 10 mM TEAA buffer pH 7.2. This material is now either used as primer for the Sanger DNA sequencing reactions or one or more glycine residues (or other suitable protected amino acid active esters) are added to create a series of mass-modified primer oligonucleotides suitable for Sanger DNA or RNA sequencing

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Immobilization of these mass-modified nucleic acid primers UP^{0-i} after primer-extension during the sequencing process can be achieved as described, e.g., in EXAMPLES 1 to 4.

EXAMPLE 6

5

Synthesis of nucleic acid primers mass-modified at C-5 of the heterocyclic base of a pyrimidine nucleoside with glycine residues

Starting material was 5-(3-aminopropynyl-1)-3' 5'-di-p-tolyldeoxyuridine prepared and 3' 5'-de-O-acylated according to literature procedures (Haralambidis *et al.*, Nucleic Acids Res. 15, 4857-76 (1987)). 0.281 g (1.0 mmole) 5-(3-aminopropynyl-1)-2'-deoxyuridine were reacted with 0.927 g (2.0 mmole) N-Fmoc-glycine pentafluorophenylester in 5 ml absolute N,N-dimethylformamide in the presence of 0.129 g (1 mmole; 174 μ l) N,N-diisopropylethylamine for 60 min at room temperature. Solvents were removed by rotary evaporation and the product was purified by silica gel chromatography (Kieselgel 60, Merck; column: 2.5x 50 cm, elution with chloroform/methanol mixtures). Yield was 0.44 g (0.78 mmole, 78 %). In order to add another glycine residue, the Fmoc group is removed with a 20 min treatment with 20% solution of piperidine in DMF, evaporated *in vacuo* and the remaining solid material extracted three times with 20 ml ethylacetate. After having removed the remaining ethylacetate, N-Fmoc-glycine pentafluorophenylester is coupled as described above. 5-(3-(N-Fmoc-glycyl)-amidopropynyl-1)-2'-deoxyuridine is transformed into the 5'-O-dimethoxytritylated nucleoside-3'-O- β -cyanoethyl-N,N-diisopropylphosphoamidite and incorporated into automated oligonucleotide synthesis by standard procedures (H. Köster *et al.*, Nucleic Acids Res. 12, 2261 (1984)). This glycine modified thymidine analogue building block for chemical DNA synthesis can be used to substitute one or more of the thymidine/uridine nucleotides in the nucleic acid primer sequence. The Fmoc group is removed at the end of the solid phase synthesis with a 20 min treatment with a 20 % solution of piperidine in DMF at room temperature. DMF is removed by a washing step with acetonitrile and the oligonucleotide deprotected and purified in the standard way

30

EXAMPLE 7

Synthesis of a nucleic acid primer mass-modified at C-5 of the heterocyclic base of a pyrimidine nucleoside with β -alanine residues

- Starting material was the same as in EXAMPLE 6. 0.281 g (1.0 mmole)
- 5 5-(3-Aminopropynyl)-2'-deoxyuridine was reacted with N-Fmoc- β -alanine pentafluorophenylester (0.955 g, 2.0 mmole) in 5 ml N,N-dimethylformamide (DMF) in the presence of 0.129 g (174 μ l; 1.0 mmole) N,N-disopropylethylamine for 60 min at room temperature. Solvents were removed and the product purified by silica gel chromatography as described in EXAMPLE 6. Yield was 0.425 g (0.74 mmole, 74 %).
- 10 Another β -alanine moiety can be added in exactly the same way after removal of the Fmoc group. The preparation of the 5'-O-dimethoxytritylated nucleoside-3'-O- β -cyanoethyl-N,N-diisopropylphosphoramidite from 5-(3-(N-Fmoc- β -alanyl)-amidopropynyl)-2'-deoxyuridine and incorporation into automated oligonucleotide synthesis is performed under standard conditions. This building block can substitute for
- 15 any of the thymidine/uridine residues in the nucleic acid primer sequence. In the case of only one incorporated mass-modified nucleotide, the nucleic acid primer molecules prepared according to EXAMPLES 6 and 7 would have a mass difference of 14 daltons.

EXAMPLE 8

Synthesis of a nucleic acid primer mass-modified at C-5 of the heterocyclic base of a pyrimidine nucleoside with ethylene glycol monomethyl ether

5 As a nucleosidic component, 5-(3-aminopropynyl-1)-2'-deoxyuridine was used in this example (see EXAMPLES 6 and 7). The mass-modifying functionality was obtained as follows: 7.61 g (100.0 mmole) freshly distilled ethylene glycol monomethyl ether dissolved in 50 ml absolute pyridine was reacted with 10.01 g (100.0 mmole) recrystallized succinic anhydride in the presence of 1.22 g (10.0 mmole) 4-N,N-

10 dimethylaminopyridine overnight at room temperature. The reaction was terminated by the addition of water (5.0 ml), the reaction mixture evaporated *in vacuo*, co-evaporated twice with dry toluene (20 ml each) and the residue redissolved in 100 ml dichloromethane. The solution was extracted successively, twice with 10 % aqueous citric acid (2 x 20 ml) and once with water (20 ml) and the organic phase dried over

15 anhydrous sodium sulfate. The organic phase was evaporated *in vacuo*, the residue redissolved in 50 ml dichloromethane and precipitated into 500 ml pentane and the precipitate dried *in vacuo*. Yield was 13.12 g (74.0 mmole; 74 %). 8.86 g (50.0 mmole) of succinylated ethylene glycol monomethyl ether was dissolved in 100 ml dioxane containing 5% dry pyridine (5 ml) and 6.96 g (50.0 mmole) 4-nitrophenol and 10.32 g

20 (50.0 mmole) dicyclohexylcarbodiimide was added and the reaction run at room temperature for 4 hours. Dicyclohexylurea was removed by filtration, the filtrate evaporated *in vacuo* and the residue redissolved in 50 ml anhydrous DMF. 12.5 ml (about 12.5 mmole 4-nitrophenylester) of this solution was used to dissolve 2.81 g (10.0 mmole) 5-(3-aminopropynyl-1)-2'-deoxyuridine. The reaction was performed in the

25 presence of 1.01 g (10.0 mmole; 1.4 ml) triethylamine at room temperature overnight. The reaction mixture was evaporated *in vacuo*, co-evaporated with toluene, redissolved in dichloromethane and chromatographed on silicagel (Si60, Merck; column 4x50 cm) with dichloromethane/methanol mixtures. The fractions containing the desired compound were collected, evaporated, redissolved in 25 ml dichloromethane and precipitated into

30 250 ml pentane. The dried precipitate of 5-(3-N-(O-succinyl ethylene glycol monomethyl ether)-amidopropynyl-1)-2'-deoxyuridine (yield: 65 %) is 5'-O-dimethoxytritylated and

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transformed into the nucleoside-3'-O-β-cyanoethyl-N, N-diisopropylphosphoamidite and incorporated as a building block in the automated oligonucleotide synthesis according to standard procedures. The mass-modified nucleotide can substitute for one or more of the thymidine/uridine residues in the nucleic acid primer sequence. Deprotection and
5 purification of the primer oligonucleotide also follows standard procedures.

EXAMPLE 9**Synthesis of a nucleic acid primer mass-modified at C-5 of the heterocyclic base of a pyrimidine nucleoside with diethylene glycol monomethyl ether**

5 Nucleosidic starting material was as in previous examples, 5-(3-aminopropynyl-1)-2'-deoxyuridine. The mass-modifying functionality was obtained similar to EXAMPLE 8. 12.02 g (100.0 mmole) freshly distilled diethylene glycol monomethyl ether dissolved in 50 ml absolute pyridine was reacted with 10.01 g (100.0 mmole) recrystallized succinic anhydride in the presence of 1.22 g (10.0 mmole) 4-N, N-dimethylaminopyridine (DMAP) overnight at room temperature. The work-up was as described in EXAMPLE 8. Yield was 18.35 g (82.3 mmole, 82.3 %). 11.06 g (50.0 mmole) of succinylated diethylene glycol monomethyl ether was transformed into the 4-nitrophenylester and, subsequently, 12.5 mmole was reacted with 2.81 g (10.0 mmole) of 5-(3-aminopropynyl-1)-2'-deoxyuridine as described in EXAMPLE 8. Yield after silica gel column chromatography and precipitation into pentane was 3.34 g (6.9 mmole, 69 %). After dimethoxytritylation and transformation into the nucleoside- β -cyanoethylphosphoramidite, the mass-modified building block is incorporated into automated chemical DNA synthesis according to standard procedures. Within the sequence of the nucleic acid primer UP^{0-i} , one or more of the thymidine/uridine residues can be substituted by this mass-modified nucleotide. In the case of only one incorporated mass-modified nucleotide, the nucleic acid primers of EXAMPLES 8 and 9 would have a mass difference of 44.05 daltons

EXAMPLE 10

25

Synthesis of a nucleic acid primer mass-modified at C-8 of the heterocyclic base of deoxyadenosine with glycine

Starting material was N^6 -benzoyl-8-bromo-5'-O-(4,4'-dimethoxytrityl)-2'-deoxyadenosine prepared according to literature (Singh *et al.*, Nucleic Acids Res. **18**, 3339-45 (1990)). 632.5 mg (1.0 mmole) of this 8-bromo-deoxyadenosine derivative was suspended in 5 ml absolute ethanol and reacted with 251.2 mg (2.0 mmole) glycine

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methyl ester (hydrochloride) in the presence of 241.4 mg (2.1 mmole; 366 μ l) N, N-diisopropylethylamine and refluxed until the starting nucleosidic material had disappeared (4-6 hours) as checked by thin layer chromatography (TLC). The solvent was evaporated and the residue purified by silica gel chromatography (column 2.5x50 cm) using solvent mixtures of chloroform/methanol containing 0.1 % pyridine. The product fractions were combined, the solvent evaporated, the fractions dissolved in 5 ml dichloromethane and precipitated into 100 ml pentane. Yield was 487 mg (0.76 mmole, 76 %). Transformation into the corresponding nucleoside- β -cyanoethylphosphoamidite and integration into automated chemical DNA synthesis is performed under standard conditions. During final deprotection with aqueous concentrated ammonia, the methyl group is removed from the glycine moiety. The mass-modified building block can substitute one or more deoxyadenosine/adenosine residues in the nucleic acid primer sequence.

EXAMPLE 11

Synthesis of a nucleic acid primer mass-modified at C-8 of the heterocyclic base of deoxyadenosine with glycylglycine

This derivative was prepared in analogy to the glycine derivative of
EXAMPLE 10. 632.5 mg (1.0 mmole) N⁶-Benzoyl-8-bromo-5'-O-(4,4'-dimethoxytrityl)-2'-deoxyadenosine was suspended in 5 ml absolute ethanol and reacted with 324.3 mg (2.0 mmole) glycyl-glycine methyl ester in the presence of 241.4 mg (2.1 mmole, 366 μ l) N, N-diisopropylethylamine. The mixture was refluxed and completeness of the reaction checked by TLC. Work-up and purification was similar to that described in EXAMPLE 10. Yield after silica gel column chromatography and precipitation into pentane was 464 mg (0.65 mmole, 65 %). Transformation into the nucleoside- β -cyanoethylphosphoamidite and into synthetic oligonucleotides is done according to standard procedures. In the case where only one of the deoxyadenosine/adenosine residues in the nucleic acid primer is substituted by this mass-modified nucleotide, the

mass difference between the nucleic acid primers of EXAMPLES 10 and 11 is 57.03 daltons

EXAMPLE 12

5 **Synthesis of a nucleic acid primer mass-modified at the C-2' of the sugar moiety of 2'-amino-2'-deoxythymidine with ethylene glycol monomethyl ether residues**

Starting material was 5'-O-(4,4-dimethoxytrityl)-2'-amino-2'-

deoxythymidine synthesized according to published procedures (e.g., Verheyden *et al.*, 1
Org. Chem. 36, 250-254 (1971); Sasaki *et al.*, 1 Org. Chem. 41, 3138-3143 (1976);
10 Imazawa *et al.*, 1 Org. Chem. 44, 2039-2041 (1979); Hobbs *et al.*, 1 Org. Chem. 42,
714-719 (1976); Ikehara *et al.*, Chem. Pharm. Bull. Japan 26, 240-244 (1978); see also
PCT Application WO 88/00201). 5'-O-(4,4-Dimethoxytrityl)-2'-amino-2'-
deoxythymidine (559.62 mg; 1.0 mmole) was reacted with 2.0 mmole of the 4-
nitrophenyl ester of succinylated ethylene glycol monomethyl ether (see EXAMPLE 8) in
15 10 ml dry DMF in the presence of 1.0 mmole (140 μ l) triethylamine for 18 hours at room
temperature. The reaction mixture was evaporated *in vacuo*, co-evaporated with
toluene, redissolved in dichloromethane and purified by silica gel chromatography (Si60,
Merck; column: 2.5x50 cm; eluent: chloroform/methanol mixtures containing 0.1 %
triethylamine). The product containing fractions were combined, evaporated and
20 precipitated into pentane. Yield was 524 mg (0.73 mmol, 73 %). Transformation into
the nucleoside- β -cyanoethyl-N,N-diisopropylphosphoamidite and incorporation into the
automated chemical DNA synthesis protocol is performed by standard procedures. The
mass-modified deoxythymidine derivative can substitute for one or more of the thymidine
residues in the nucleic acid primer.

25 In an analogous way, by employing the 4-nitrophenyl ester of succinylated
diethylene glycol monomethyl ether (see EXAMPLE 9) and triethylene glycol
monomethyl ether, the corresponding mass-modified oligonucleotides are prepared. In
the case of only one incorporated mass-modified nucleoside within the sequence, the
mass difference between the ethylene, diethylene and triethylene glycol derivatives is
30 44.05, 88.1 and 132.15 daltons respectively.

EXAMPLE 13**Synthesis of a nucleic acid primer mass-modified in the internucleotidic linkage via alkylation of phosphorothioate groups**

5 Phosphorothioate-containing oligonucleotides were prepared according to standard procedures (see e.g. Gait *et al.*, Nucleic Acids Res., 19 1183 (1991)). One, several or all internucleotide linkages can be modified in this way. The (-)-M13 nucleic acid primer sequence (17-mer) 5'-dGTAAAACGACGGCCAGT was synthesized in 0.25 μ mole scale on a DNA synthesizer and one phosphorothioate group introduced after the
10 final synthesis cycle (G to T coupling). Sulfurization, deprotection and purification followed standard protocols. Yield was 31.4 nmole (12.6 % overall yield), corresponding to 31.4 nmole phosphorothioate groups. Alkylation was performed by dissolving the residue in 31.4 μ l TE buffer (0.01 M Tris pH 8.0, 0.001 M EDTA) and by adding 16 μ l of a solution of 20 mM solution of 2-iodoethanol (320 nmole; i.e., 10-fold
15 excess with respect to phosphorothioate diesters) in N,N-dimethylformamide (DMF). The alkylated oligonucleotide was purified by standard reversed phase HPLC (RP-18 Ultraphere, Beckman; column: 4.5 x 250 mm; 100 mM triethylammonium acetate, pH 7.0 and a gradient of 5 to 40 % acetonitrile).

 In a variation of this procedure, the nucleic acid primer containing one or
20 more phosphorothioate phosphodiester bond is used in the Sanger sequencing reactions. The primer-extension products of the four sequencing reactions are purified as exemplified in EXAMPLES 1 - 4, cleaved off the solid support, lyophilized and dissolved in 4 μ l each of TE buffer pH 8.0 and alkylated by addition of 2 μ l of a 20 mM solution of 2-iodoethanol in DMF. It is then analyzed by ES and/or MALDI mass spectrometry.

25 In an analogous way, employing instead of 2-iodoethanol, e.g., 3-iodopropanol, 4-iodobutanol mass-modified nucleic acid primer are obtained with a mass difference of 14.03, 28.06 and 42.03 daltons respectively compared to the unmodified phosphorothioate phosphodiester-containing oligonucleotide.

EXAMPLE 14

Synthesis of 2'-amino-2'-deoxyuridine-5'-triphosphate and 3'-amino-2',3'-dideoxythymidine-5'-triphosphate mass-modified at the 2'- or 3'-amino function with glycine or β -alanine residues

Starting material was 2'-azido-2'-deoxyuridine prepared according to literature (Verheyden *et al.*, J. Org. Chem. **36**, 250 (1971)), which was 4,4-dimethoxytritylated at 5'-OH with 4,4-dimethoxytrityl chloride in pyridine and acetylated at 3'-OH with acetic anhydride in a one-pot reaction using standard reaction conditions. With 191 mg (0.71 mmole) 2'-azido-2'-deoxyuridine as starting material, 396 mg (0.65 mmol, 90.8 %) 5'-O-(4,4-dimethoxytrityl)-3'-O-acetyl-2'-azido-2'-deoxyuridine was obtained after purification via silica gel chromatography. Reduction of the azido group was performed using published conditions (Barta *et al.*, Tetrahedron **46**, 587-594 (1990)). Yield of 5'-O-(4,4-dimethoxytrityl)-3'-O-acetyl-2'-amino-2'-deoxyuridine after silica gel chromatography was 288 mg (0.49 mmole; 76 %). This protected 2'-amino-2'-deoxyuridine derivative (588 mg, 1.0 mmole) was reacted with 2 equivalents (927 mg, 2.0 mmole) N-Fmoc-glycine pentafluorophenyl ester in 10 ml dry DMF overnight at room temperature in the presence of 1.0 mmole (174 μ l) N,N-diisopropylethylamine. Solvents were removed by evaporation *in vacuo* and the residue purified by silica gel chromatography. Yield was 711 mg (0.71 mmole, 82 %). Detritylation was achieved by a one hour treatment with 80% aqueous acetic acid at room temperature. The residue was evaporated to dryness, co-evaporated twice with toluene, suspended in 1 ml dry acetonitrile and 5'-phosphorylated with POCl₃ according to literature (Yoshikawa *et al.*, Bull. Chem. Soc. Japan **42**, 3505 (1969) and Sowa *et al.*, Bull. Chem. Soc. Japan **48**, 2084 (1975)) and directly transformed in a one-pot reaction to the 5'-triphosphate using 3 ml of a 0.5 M solution (1.5 mmole) tetra (tri-n-butylammonium) pyrophosphate in DMF according to literature (e.g. Seela *et al.*, Helvetica Chimica Acta **74**, 1048 (1991)). The Fmoc and the 3'-O-acetyl groups were removed by a one-hour treatment with concentrated aqueous ammonia at room temperature and the reaction mixture evaporated and lyophilized. Purification also followed standard procedures by using anion-exchange chromatography on DEAE-Sephadex with a linear gradient of triethylammonium bicarbonate (0.1 M - 1.0 M). Triphosphate containing fractions (checked by thin layer

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chromatography on polyethylencimine cellulose plates) were collected, evaporated and lyophilized. Yield (by UV-absorbance of the uracil moiety) was 68% (0.48 mmole).

A glycyl-glycine modified 2'-amino-2'-deoxyuridine-5'-triphosphate was obtained by removing the Fmoc group from 5'-O-(4,4-dimethoxytrityl)-3'-O-acetyl-2'-N-(N-9-fluorenylmethoxycarbonyl-glycyl)-2'-amino-2'-deoxyuridine by a one-hour treatment with a 20% solution of piperidine in DMF at room temperature, evaporation of solvents, two-fold co-evaporation with toluene and subsequent condensation with N-Fmoc-glycine pentafluorophenyl ester. Starting with 1.0 mmole of the 2'-N-glycyl-2'-amino-2'-deoxyuridine derivative and following the procedure described above, 0.72 mmole (72%) of the corresponding 2'-(N-glycyl-glycyl)-2'-amino-2'-deoxyuridine-5'-triphosphate was obtained.

Starting with 5'-O-(4,4-dimethoxytrityl)-3'-O-acetyl-2'-amino-2'-deoxyuridine and coupling with N-Fmoc-β-alanine pentafluorophenyl ester, the corresponding 2'-(N-β-alanyl)-2'-amino-2'-deoxyuridine-5'-triphosphate can be synthesized. These modified nucleoside triphosphates are incorporated during the Sanger DNA sequencing process in the primer-extension products. The mass difference between the glycine, β-alanine and glycyl-glycine mass-modified nucleosides is, per nucleotide incorporated, 58.06, 72.09 and 115.1 daltons respectively.

When starting with 5'-O-(4,4-dimethoxytrityl)-3'-amino-2',3'-dideoxythymidine (obtained by published procedures, see EXAMPLE 12), the corresponding 3'-(N-glycyl)-3'-amino-/ 3'-(N-glycyl-glycyl)-3'-amino-/ and 3'-(N-β-alanyl)-3'-amino-2',3'-dideoxythymidine-5'-triphosphates can be obtained. These mass-modified nucleoside triphosphates serve as a terminating nucleotide unit in the Sanger DNA sequencing reactions providing a mass difference per terminated fragment of 58.06, 72.09 and 115.1 daltons respectively when used in the multiplexing sequencing mode. The mass-differentiated fragments can then be analyzed by ES and/or MALDI mass spectrometry.

EXAMPLE 15

Synthesis of deoxyuridine-5'-triphosphate mass-modified at C-5 of the heterocyclic base with glycine, glycyl-glycine and β -alanine residues.

0.281 g (1.0 mmole) 5-(3-Aminopropynyl-1)-2'-deoxyuridine (see EXAMPLE 6) was reacted with either 0.927 g (2.0 mmole) N-Fmoc-glycine pentafluorophenylester or 0.955 g (2.0 mmole) N-Fmoc- β -alanine pentafluorophenyl ester in 5 ml dry DMF in the presence of 0.129 g N, N-diisopropylethylamine (174 μ l, 1.0 mmole) overnight at room temperature. Solvents were removed by evaporation *in vacuo* and the condensation products purified by flash chromatography on silica gel (Still *et al.*, J. Org. Chem. **43**, 2923-2925 (1978)). Yields were 476 mg (0.85 mmole; 85%) for the glycine and 436 mg (0.76 mmole; 76%) for the β -alanine derivatives. For the synthesis of the glycyl-glycine derivative, the Fmoc group of 1.0 mmole Fmoc-glycine-deoxyuridine derivative was removed by one-hour treatment with 20% piperidine in DMF at room temperature. Solvents were removed by evaporation *in vacuo*, the residue was co-evaporated twice with toluene and condensed with 0.927 g (2.0 mmole) N-Fmoc-glycine pentafluorophenyl ester and purified as described above. Yield was 445 mg (0.72 mmole; 72%). The glycyl-, glycyl-glycyl- and β -alanyl-2'-deoxyuridine derivatives, N-protected with the Fmoc group were transformed to the 3'-O-acetyl derivatives by tritylation with 4,4-dimethoxytrityl chloride in pyridine and acetylation with acetic anhydride in pyridine in a one-pot reaction and subsequently detritylated by one hour treatment with 80% aqueous acetic acid according to standard procedures. Solvents were removed, the residues dissolved in 100 ml chloroform and extracted twice with 50 ml 10% sodium bicarbonate and once with 50 ml water, dried with sodium sulfate, the solvent evaporated and the residues purified by flash chromatography on silica gel. Yields were 361 mg (0.60 mmole; 71%) for the glycyl-, 351 mg (0.57 mmole; 75%) for the β -alanyl- and 323 mg (0.49 mmole; 68%) for the glycyl-glycyl-3'-O'-acetyl-2'-deoxyuridine derivatives respectively. Phosphorylation at the 5'-OH with POCl₃, transformation into the 5'-triphosphate by *in-situ* reaction with tetra(tri-n-butylammonium) pyrophosphate in DMF, 3'-de-O-acetylation, cleavage of the Fmoc group, and final purification by anion-exchange chromatography on DEAE-Sephadex was performed as described in EXAMPLE 14. Yields according to UV-absorbance of the uracil moiety were 0.41 mmole 5-(3-(N-glycyl)-amidopropynyl-1)-2'-deoxyuridine-5'-triphosphate (84%), 0.43 mmole 5-(3-(N- β -

alanyl)-amidopropynyl-1)-2'-deoxyuridine-5'-triphosphate (75%) and 0.38 mmole 5-(3-(N-glycyl-glycyl)-amidopropynyl-1)-2'-deoxyuridine-5'-triphosphate (78%).

These mass-modified nucleoside triphosphates were incorporated during the Sanger DNA sequencing primer-extension reactions.

5 When using 5-(3-aminopropynyl-1)-2',3'-dideoxyuridine as starting material and following an analogous reaction sequence the corresponding glycyl-, glycyl-glycyl- and β -alanyl-2',3'-dideoxyuridine-5'-triphosphates were obtained in yields of 69, 63 and 71% respectively. These mass-modified nucleoside triphosphates serve as chain-terminating nucleotides during the Sanger DNA sequencing reactions. The mass-
10 modified sequencing ladders are analyzed by either ES or MALDI mass spectrometry.

EXAMPLE 16

Synthesis of 8-glycyl- and 8-glycyl-glycyl-2'-deoxyadenosine-5'-triphosphate

15 727 mg (1.0 mmole) of N⁶-(4-tert-butylphenoxyacetyl)-8-glycyl-5'-(4,4-dimethoxytrityl)-2'-deoxyadenosine or 800 mg (1.0 mmole) N⁶-(4-tert-butylphenoxyacetyl)-8-glycyl-glycyl-5'-(4,4-dimethoxytrityl)-2'-deoxyadenosine prepared according to EXAMPLES 10 and 11 and literature (Köster *et al.*, **Tetrahedron** 37, 362 (1981)) were acetylated with acetic anhydride in pyridine at the 3'-OH, detritylated at the
20 5'-position with 80% acetic acid in a one-pot reaction and transformed into the 5'-triphosphates via phosphorylation with POCl₃ and reaction *in-situ* with tetra(tri-n-butylammonium) pyrophosphate as described in EXAMPLE 14. Deprotection of the N⁶-tert-butylphenoxyacetyl, the 3'-O-acetyl and the O-methyl group at the glycine residues was achieved with concentrated aqueous ammonia for ninety minutes at room
25 temperature. Ammonia was removed by lyophilization and the residue washed with dichloromethane, solvent removed by evaporation *in vacuo* and the remaining solid material purified by anion-exchange chromatography on DEAE-Sephadex using a linear gradient of triethylammonium bicarbonate from 0.1 to 1.0 M. The nucleoside triphosphate containing fractions (checked by TLC on polyethyleneimine cellulose plates)
30 were combined and lyophilized. Yield of the 8-glycyl-2'-deoxyadenosine-5'-triphosphate

(determined by UV-absorbance of the adenine moiety) was 57% (0.57 mmole). The yield for the 8-glycyl-glycyl-2'-deoxyadenosine-5'-triphosphate was 51% (0.51 mmole).

These mass-modified nucleoside triphosphates were incorporated during primer-extension in the Sanger DNA sequencing reactions.

5 When using the corresponding N6-(4-tert-butylphenoxyacetyl)-8-glycyl- or -glycyl-glycyl-5'-O-(4,4-dimethoxytrityl)-2',3'-dideoxyadenosine derivatives as starting materials prepared according to standard procedures (see, e.g., for the introduction of the 2',3'-function: Seela *et al.*, Helvetica Chimica Acta **74**, 1048-1058 (1991)) and using an analogous reaction sequence as described above, the chain-terminating mass-modified nucleoside triphosphates 8-glycyl- and 8-glycyl-glycyl-2',3'-dideoxyadenosine-5'-triphosphates were obtained in 53 and 47% yields respectively. The mass-modified sequencing fragment ladders are analyzed by either ES or MALDI mass spectrometry.

EXAMPLE 17

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Mass-modification of Sanger DNA sequencing fragment ladders by incorporation of chain-elongating 2'-deoxy- and chain-terminating 2',3'-dideoxythymidine-5'-(alpha-S)-triphosphate and subsequent alkylation with 2-iodoethanol and 3-iodopropanol

20 2',3'-Dideoxythymidine-5'-(alpha-S)-triphosphate was prepared according to published procedures (e.g., for the alpha-S-triphosphate moiety: Eckstein *et al.*, Biochemistry **15**, 1685 (1976) and Accounts Chem. Res. **12**, 204 (1978) and for the 2',3'-dideoxy moiety: Seela *et al.*, Helvetica Chimica Acta, **74**, 1048-1058 (1991)). Sanger DNA sequencing reactions employing 2'-deoxythymidine-5'-(alpha-S)-triphosphate are performed according to standard protocols (e.g. Eckstein, Ann. Rev. Biochem. **54**, 367 25 (1985)). When using 2',3'-dideoxythymidine-5'-(alpha-S)-triphosphates, this is used instead of the unmodified 2',3'-dideoxythymidine-5'-triphosphate in standard Sanger DNA sequencing (see e.g. Swerdlow *et al.*, Nucleic Acids Res. **18**, 1415-1419 (1990)). The template (2 pmole) and the nucleic acid M13 sequencing primer (4 pmole) modified 30 according to EXAMPLE 1 are annealed by heating to 65°C in 100 µl of 10 mM Tris-HCl pH 7.5, 10 mM MgCl₂, 50 mM NaCl, 7 mM dithiothreitol (DTT) for 5 min and slowly

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brought to 37°C during a one hour period. The sequencing reaction mixtures contain, as exemplified for the T-specific termination reaction, in a final volume of 150 µl, 200 µM (final concentration) each of dATP, dCTP, dTTP, 300 µM ε7-deaza-dGTP, 5 µM 2',3'-dideoxythymidine-5'-(α-S)-triphosphate and 40 units Sequenase (United States Biochemicals). Polymerization is performed for 10 min at 37°C, the reaction mixture heated to 70°C to inactivate the Sequenase, ethanol precipitated and coupled to thiolated Sequelon membrane disks (8 mm diameter) as described in EXAMPLE 1. Alkylation is performed by treating the disks with 10 µl of 10 mM solution of either 2-iodoethanol or 3-iodopropanol in NMM (N-methylmorpholine/water/2-propanol, 2/49/49, v/v/v) (three times), washing with 10 µl NMM (three times) and cleaving the alkylated T-terminated primer-extension products off the support by treatment with DTT as described in EXAMPLE 1. Analysis of the mass-modified fragment families is performed with either ES or MALDI mass spectrometry.

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EXAMPLE 18

Analysis of a Mixture of Oligothymidylic Acids

Oligothymidylic acid, oligo p(dT)₁₂₋₁₈, is commercially available (United States Biochemical, Cleveland, OH). Generally, a matrix solution of 0.5 M in ethanol was prepared. Various matrices were used for this Example and Examples 19- 21 such as 3,5-dihydroxybenzoic acid, sinapinic acid, 3-hydroxypicolinic acid, 2,4,6-trihydroxyacetophenone. Oligonucleotides were lyophilized after purification by HPLC and taken up in ultrapure water (MilliQ, Millipore) using amounts to obtain a concentration of 10 pmoles/µl as stock solution. An aliquot (1 µl) of this concentration or a dilution in ultrapure water was mixed with 1 µl of the matrix solution on a flat metal surface serving as the probe tip and dried with a fan using cold air. In some experiments, cation-ion exchange beads in the acid form were added to the mixture of matrix and sample solution.

MALDI-TOF spectra were obtained for this Example and Examples 19-21 on different commercial instruments such as Vision 2000 (Finnigan-MAT), VG ToFSpec (Fisons Instruments), LaserTec Research (Vestec). The conditions for this Example were

30

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linear negative ion mode with an acceleration voltage of 25 kV. The MALDI-TOF spectrum generated is shown in FIGURE 14. Mass calibration was done externally and generally achieved by using defined peptides of appropriate mass range such as insulin, gramicidin S, trypsinogen, bovine serum albumen, and cytochrome C. All spectra were
5 generated by employing a nitrogen laser with 5 nsec pulses at a wavelength of 337 nm. Laser energy varied between 10^6 and 10^7 W/cm². To improve signal-to-noise ratio generally, the intensities of 10 to 30 laser shots were accumulated.

EXAMPLE 19**Mass Spectrometric Analysis of a 50-mer and a 99-mer**

Two large oligonucleotides were analyzed by mass spectrometry. The 50-mer
5 d (TAACGGTCATTACGGCCATTGACTGTAGGACCTGCATTACATGACTAGCT)
(SEQ ID NO:3) and dT(pdT)₉₉ were used. The oligodeoxynucleotides were synthesized
using β -cyanoethylphosphoramidites and purified using published procedures.(e.g. N.D.
Sinha, J. Biernat, J. McManus and H. Köster, *Nucleic Acids Res.* **12**, 4539 (1984))
10 employing commercially available DNA synthesizers from either Millipore (Bedford, MA)
or Applied Biosystems (Foster City, CA) and HPLC equipment and RP18 reverse phase
columns from Waters (Milford, MA) The samples for mass spectrometric analysis were
prepared as described in Example 18. The conditions used for MALDI-MS analysis of
each oligonucleotide were 500 fmol of each oligonucleotide, reflectron positive ion mode
15 with an acceleration of 5 kV and postacceleration of 20 kV. The MALDI-TOF spectra
generated were superimposed and are shown in FIGURE 15.

EXAMPLE 20**Simulation of the DNA Sequencing Results of FIGURE 2**

The 13 DNA sequences representing the nested dT-terminated fragments
of the Sanger DNA sequencing for the 50-mer described in Example 19 (SEQ ID NO:3)
were synthesized as described in Example 19. The samples were treated and 500 fmol of
each fragment was analyzed by MALDI-MS as described in Example 18. The resulting
25 MALDI-TOF spectra are shown in FIGURE 16. The conditions were reflectron positive
ion mode with an acceleration of 5 kV and postacceleration of 20 kV. Calculated
molecular masses and experimental molecular masses are shown in Table I

The MALDI-TOF spectra were superimposed (FIGURE 17) to
demonstrate that the individual peaks are resolvable even between the 10-mer and 11-mer
30 (upper panel) and the 37-mer and 38-mer (lower panel). The two panels show two
different scales and the spectra analyzed at that scale

EXAMPLE 21**MALDI-MS Analysis of a Mass-Modified Oligonucleotide**

- 5 A 17-mer was mass-modified at C-5 of one or two deoxyuridine moieties.
5-[13-(2-Methoxyethoxyl)-tridecynyl]-5'-O-(4,4'-dimethoxytrityl)-2'-deoxyuridine-3'-
β-cyanoethyl-N, N-diisopropylphosphoramidite was used to synthesize the modified 17-
mers using the methods described in Example 19.

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The modified 17-mers were

X

|

a: d (TAAAACGACGGCCAGUG) (molecular mass: 5454)
(SEQ ID NO:4)

X

|

X

|

b: d (UAAAACGACGGCCAGUG) (molecular mass 5634)
(SEQ ID NO:5)

where X = -C≡C-(CH₂)₁₁-OH

(unmodified 17-mer: molecular mass: 5273)

The samples were prepared and 500 fmol of each modified 17-mer was analyzed using MALDI-MS as described in Example 18. The conditions used were reflectron positive ion mode with an acceleration of 5 kV and postacceleration of 20 kV. The MALDI-TOF spectra which were generated were superimposed and are shown in FIGURE 18.

EXAMPLE 22

Detection of Polymerase Chain Reaction Products Containing 7-Deazapurine

MATERIALS AND METHODS

PCR amplifications

The following oligodeoxynucleotide primers were either synthesized according to standard phosphoramidite chemistry (Sinha, N.D., et al., (1983) *Tetrahedron Let.* V 1. 24, Pp. 5843-5846; Sinha, N.D., et al., (1984) *Nucleic Acids Res.*, Vol. 12, Pp. 4539-4557) on a MilliGen 7500 DNA synthesizer (Millipore, Bedford, MA, USA) in 200

nmol scales or purchased from MWG-Biotech (Ebersberg, Germany, primer 3) and Biometra (Göttingen, Germany, primers 6-7).

- primer 1: 5' - GTCACCCTCGACCTGCAG (SEQ. ID. NO. 6);
 5 primer 2: 5' - TTGTAAAACGACGGCCAGT (SEQ. ID. NO. 7);
 primer 3: 5' - CTTCCACCGCGATGTTGA (SEQ. ID. NO. 8);
 primer 4: 5' - CAGGAAACAGCTATGAC (SEQ. ID. NO. 9);
 primer 5: 5' - GTAAAACGACGGCCAGT (SEQ. ID. NO. 10);
 primer 6: 5' - GTCACCCTCGACCTGCAGC (g: RiboG) (SEQ. ID. NO. 11);
 10 primer 7: 5' - GTTGTAAPACGAGGGCCAGT (g: RiboG) (SEQ. ID. NO. 12).

The 99-mer and 200-mer DNA strands (modified and unmodified) as well as the ribo- and 7-deaza-modified 100-mer were amplified from pRFc1 DNA (10 ng, generously supplied S. Feyerabend, University of Hamburg) in 100 µL reaction volume containing 10 mmol/L KCl, 10 mmol/L (NH₄)₂SO₄, 20 mmol/L Tris HCl (pH = 8.8), 2
 15 mmol/L MgSO₄, (exo(-))*Pseudococcus furiosus* (*Pfu*) -Buffer, Pharmacia, Freiburg, Germany), 0.2 mmol/L each dNTP (Pharmacia, Freiburg, Germany), 1 µmol/L of each primer and 1 unit of exo(-)*Pfu* DNA polymerase (Stratagene, Heidelberg, Germany)

For the 99-mer primers 1 and 2, for the 200-mer primers 1 and 3 and for the 100-mer primers 6 and 7 were used. To obtain 7-deazapurine modified nucleic acids,
 20 during PCR-amplification dATP and dGTP were replaced with 7-deaza-dATP and 7-deaza-dGTP. The reaction was performed in a thermal cycler (OmniGene, MWG-Biotech, Ebersberg, Germany) using the cycle: denaturation at 95°C for 1 min., annealing at 51°C for 1 min. and extension at 72°C for 1 min. For all PCRs the number of reaction cycles was 30. The reaction was allowed to extend for additional 10 min. at 72°C after
 25 the last cycle.

The 103-mer DNA strands (modified and unmodified) were amplified from M13mp18 RFI DNA (100 ng, Pharmacia, Freiburg, Germany) in 100 µL reaction volume using primers 4 and 5 all other concentrations were unchanged. The reaction was performed using the cycle: denaturation at 95°C for 1 min., annealing at 40°C for 1 min.
 30 and extension at 72°C for 1 min. After 30 cycles for the unmodified and 40 cycles for

- 60 -

the modified 103-mer respectively, the samples were incubated for additional 10 min. at 72°C.

Synthesis of 5'-[³²P]-labeled PCR-primers

5 Primers 1 and 4 were 5'-[³²P]-labeled employing T4-polynucleotidkinase (Epicentre Technologies) and (γ-³²P)-ATP. (BLU/NGG/502A, Dupont, Germany) according to the protocols of the manufacturer. The reactions were performed substituting 10% of primer 1 and 4 in PCR with the labeled primers under otherwise unchanged reaction-conditions. The amplified DNAs were separated by gel
10 electrophoresis on a 10% polyacrylamide gel. The appropriate bands were excised and counted on a Packard TRI-CARB 460C liquid scintillation system (Packard, CT, USA).

Primer-cleavage from ribo-modified PCR-product

15 The amplified DNA was purified using Ultrafree-MC filter units (30,000 NMWL), it was then redissolved in 100 µl of 0.2 mol/L NaOH and heated at 95°C for 25 minutes. The solution was then acidified with HCl (1 mol/L) and further purified for MALDI-TOF analysis employing Ultrafree-MC filter units (10,000 NMWL) as described below.

Purification of PCR products

20 All samples were purified and concentrated using Ultrafree-MC units 30000 NMWL (Millipore, Eschborn, Germany) according to the manufacturer's description. After lyophilisation, PCR products were redissolved in 5 µL (3 µL for the 200-mer) of ultrapure water. This analyte solution was directly used for MALDI-TOF
25 measurements.

MALDI-TOF MS

30 Aliquots of 0.5 µL of analyte solution and 0.5 µL of matrix solution (0.7 mol/L 3-HPA and 0.07 mol/L ammonium citrate in acetonitrile/water (1:1, v/v)) were mixed on a flat metallic sample support. After drying at ambient temperature the sample was introduced into the mass spectrometer for analysis. The MALDI-TOF mass

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spectrometer used was a Finnigan MAT Vision 2000 (Finnigan MAT, Bremen, Germany). Spectra were recorded in the positive ion reflector mode with a 5 keV ion source and 20 keV postacceleration. The instrument was equipped with a nitrogen laser (337 nm wavelength). The vacuum of the system was $3-4 \cdot 10^{-8}$ hPa in the analyzer region and $1-4 \cdot 10^{-7}$ hPa in the source region. Spectra of modified and unmodified DNA samples were obtained with the same relative laser power; external calibration was performed with a mixture of synthetic oligodeoxynucleotides (7-to50-mer).

RESULTS AND DISCUSSION

Enzymatic synthesis of 7-deazapurine nucleotide containing nucleic acids by PCR

In order to demonstrate the feasibility of MALDI-TOF MS for the rapid, gel-free analysis of short PCR products and to investigate the effect of 7-deazapurine modification of nucleic acids under MALDI-TOF conditions, two different primer-template systems were used to synthesize DNA fragments. Sequences are displayed in Figures 24 and 25. While the two single strands of the 103-mer PCR product had nearly equal masses ($\Delta m = 8$ u), the two single strands of the 99-mer differed by 526 u.

Considering the facts that 7-deaza purine nucleotide building blocks for chemical DNA synthesis are approximately 160 times more expensive than regular ones (Product Information, Glen Research Corporation, Sterling, VA) and their application in standard β -cyano-phosphoramidite chemistry is not trivial (Product Information, Glen Research Corporation, Sterling, VA; Schneider, K and B.T. Chait (1995) *Nucleic Acids Res.* 23, 1570) the cost of 7-deaza purine modified primers would be very high. Therefore, to increase the applicability and scope of the method, all PCRs were performed using unmodified oligonucleotide primers which are routinely available. Substituting dATP and dGTP by c^7 -dATP and c^7 -dGTP in polymerase chain reaction led to products containing approximately 80% 7-deaza-purine modified nucleosides for the 99-mer and 103-mer, and about 90% for the 200-mer, respectively. Table II shows the base composition of all PCR products.

30

TABLE II

Base composition of the 99-mer, 103-mer and 200-mer PCR amplification products
(unmodified and 7-deaza purine modified)

5	DNA- fragments ¹	C	T	A	G	c ⁷ -deaza- A	c ⁷ -deaza- G	rel. modificati on ²
	200-mers	54	34	56	56	-	-	-
	modified	54	34	6	5	50	51	90%
10	200-mer s	56	56	34	54	-	-	-
	203-mer s modified	56	56	3	4	31	50	92%
	200-mer a	28	25	24	28	-	-	-
	103-mer s modified	28	25	6	5	18	23	79%
15	103-mer s	28	24	23	28	-	-	-
	103-mer a modified	28	24	7	4	16	24	78%
	103-mer a	34	21	24	20	-	-	-
	99-mer s modified 99-	34	21	6	5	13	15	75%
20	mer s	20	24	21	34	-	-	-
	99-mer a modified 99-	20	24	3	4	18	30	87%
25	mer a							
	¹ "s" and "a" describe "sense" and "antisense" strands of the double-stranded PCR							

product.

² indicates relative modification as percentage of 7-deaza purine modified nucleotides of total amount of purine nucleotides.

30 However, it remained to be determined whether 80-90% 7-deaza-purine modification would be sufficient for accurate mass spectrometer detection. It was therefore important to determine whether all purine nucleotides could be substituted during the enzymatic amplification step. It was found that *exo(-)Pseudomonas furiosus* (Pfu) DNA polymerase indeed could accept c⁷-dATP and c⁷-dGTP in the absence of
35 unmodified purine triphosphates. However, the incorporation was less efficient leading to a lower yield of PCR product (Figure 26). Ethidium-bromide stains by intercalation with the stacked bases of the DNA-doublestrand. Therefore lower band intensities in the

ethidium-bromide stained gel might be artifacts since the modified DNA-strands do not necessarily need to give the same band intensities as the unmodified ones

To verify these results, the PCRs with [32 P]-labeled primers were
5 repeated. The autoradiogram (Figure 27) clearly shows lower yields for the modified PCR-products. The bands were excised from the gel and counted. For all PCR products the yield of the modified nucleic acids was about 50%, referring to the corresponding unmodified amplification product. Further experiments showed that *exo(-)DeepVent* and *Vent* DNA polymerase were able to incorporate c^7 -dATP and c^7 -dGTP during PCR as
10 well. The overall performance, however, turned out to be best for the *exo(-)Pfu* DNA polymerase giving least side products during amplification. Using all three polymerases, it was found that such PCRs employing c^7 -dATP and c^7 -dGTP instead of their isosteres showed less side-reactions giving a cleaner PCR-product. Decreased occurrence of amplification side products may be explained by a reduction of primer mismatches due to
15 a lower stability of the complex formed from the primer and the 7-deaza-purine containing template which is synthesized during PCR. Decreased melting point for DNA duplexes containing 7-deaza-purine have been described (Mizusawa, S. et al., (1986) *Nucleic Acids Res.*, 14, 1319-1324). In addition to the three polymerases specified above (*exo(-) Deep Vent* DNA polymerase, *Vent* DNA polymerase and *exo(-) Pfu* DNA
20 polymerase), it is anticipated that other polymerases, such as the Large Klenow fragment of *E.coli* DNA polymerase, *Sequenase*, *Taq* DNA polymerase, and *U AmpliTaq*, *AmpliTaq* or *AmpliTaq TS* DNA polymerase can be used. In addition, where RNA is the template, RNA polymerases, such as the SP6 or the T7 RNA polymerase, must be used

25 *MALDI-TOF mass spectrometry of modified and unmodified PCR products.*

The 99-mer, 103-mer and 200-mer PCR products were analyzed by
MALDI-TOF MS. Based on past experience, it was known that the degree of
depurination depends on the laser energy used for desorption and ionization of the
30 analyte. Since the influence of 7-deazapurine modification on fragmentation due to

depurination was to be investigated, all spectra were measured at the same relative laser energy.

Figures 28a and 28b show the mass spectra of the modified and unmodified 103-mer nucleic acids. In case of the modified 103-mer, fragmentation causes a broad $(M+H)^+$ signal. The maximum of the peak is shifted to lower masses so that the assigned mass represents a mean value of $(M+H)^+$ signal and signals of fragmented ions, rather than the $(M+H)^+$ signal itself. Although the modified 103-mer still contains about 20% A and G from the oligonucleotide primers, it shows less fragmentation which is featured by much more narrow and symmetric signals. Especially peak tailing on the lower mass side due to depurination, is substantially reduced. Hence, the difference between measured and calculated mass is strongly reduced although it is still below the expected mass. For the unmodified sample a $(M+H)^+$ signal of 31670 was observed, which is a 97 u or 0.3% difference to the calculated mass. While, in case of the modified sample this mass difference diminished to 10 u or 0.03% (31713 u found, 31723 u calculated). These observations are verified by a significant increase in mass resolution of the $(M+H)^+$ signal of the two signal strands ($m/\Delta m = 67$ as opposed to 18 for the unmodified sample with $\Delta m =$ full width at half maximum, fwhm). Because of the low mass difference between the two single strands (8 u) their individual signals were not resolved.

With the results of the 99 base pair DNA fragments the effects of increased mass resolution for 7-deazapurine containing DNA becomes even more evident. The two single strands in the unmodified sample were not resolved even though the mass difference between the two strands of the PCR product was very high with 526 u due to unequal distribution of purines and pyrimidines (figure 29a). In contrast to this, the modified DNA showed distinct peaks for the two single strands (figure 29b) which makes the superiority of this approach for the determination of molecular weights to gel electrophoretic methods even more profound. Although base line resolution was not obtained the individual masses were able to be assigned with an accuracy of 0.1%: $\Delta m = 27$ u for the lighter (calc. mass = 30224 u) and $\Delta m = 14$ u for the heavier strand (calc. mass = 30750 u). Again, it was found that the full width at half maximum was substantially decreased for the 7-deazapurine containing sample.

In case of both the 99-mer and 103-mer the 7-deazapurine containing nucleic acids seem to give higher sensitivity despite the fact that they still contain about 20% unmodified purine nucleotides. To get comparable signal-to-noise ratio at similar intensities for the $(M+H)^+$ signals, the unmodified 99-mer required 20 laser shots in contrast to 12 for the modified one and the 103-mer required 12 shots for the unmodified sample as opposed to three for the 7-deazapurine nucleoside-containing PCR product

Comparing the spectra of the modified and unmodified 200-mer amplicons, improved mass resolution was again found for the 7-deazapurine containing sample as well as increased signal intensities (figures 30a and 30b). While the signal of the single strands predominates in the spectrum of the modified sample the DNA-suplex and dimers of the single strands gave the strongest signal for the unmodified sample

A complete 7-deaza purine modification of nucleic acids may be achieved either using modified primers in PCR or cleaving the unmodified primers from the partially modified PCR product. Since disadvantages are associated with modified primers, as described above, a 100-mer was synthesized using primers with a ribo-modification. The primers were cleaved hydrolytically with NaOH according to a method developed earlier in our laboratory (Koester, H. et al., *Z. Physiol. Chem.* 359:1570-1589). Figures 31a and 31b display the spectra of the PCR product before and after primer cleavage. Figure 31b shows that the hydrolysis was successful: Both hydrolyzed PCR product as well as the two released primers could be detected together with a small signal from residual uncleaved 100-mer. This procedure is especially useful for the MALDI-TOF analysis of very short PCR-products since the share of unmodified purines originating from the primer increases with decreasing length of the amplified sequence.

The remarkable properties of 7-deazapurine modified nucleic acids can be explained by either more effective desorption and/or ionization, increased ion stability and/or a lower denaturation energy of the double stranded purine modified nucleic acid. The exchange of the N-7 for a methine group results in the loss of one acceptor for a hydrogen bond which influences the ability of the nucleic acid to form secondary structures due to non-Watson-Crick base pairing (Seela, F. and A. Kehne (1987) *Biochemistry*, 26, 2232-2238.), which should be a reason for better desorption during the MALDI process. In addition to this the aromatic system of 7-deazapurine has a lower

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electron density that weakens Watson-Crick base pairing resulting in a decreased melting point (Mizusawa, S. et al., (1986) *Nucleic Acids Res.*, 14, 1319-1324) of the double-strand. This effect may decrease the energy needed for denaturation of the duplex in the MALDI process. These aspects as well as the loss of a site which probably will carry a positive charge on the N-7 nitrogen renders the 7-deazapurine modified nucleic acid less polar and may promote the effectiveness of desorption.

Because of the absence of N-7 as proton acceptor and the decreased polarization of the C-N bond in 7-deazapurine nucleosides depurination following the mechanisms established for hydrolysis in solution is prevented. Although a direct correlation of reactions in solution and in the gas phase is problematic, less fragmentation due to depurination of the modified nucleic acids can be expected in the MALDI process. Depurination may either be accompanied by loss of charge which decreases the total yield of charged species or it may produce charged fragmentation products which decreases the intensity of the non fragmented molecular ion signal.

The observation of both increased sensitivity and decreased peak tailing of the $(M+H)^+$ signals on the lower mass side due to decreased fragmentation of the 7-deazapurine containing samples indicate that the N-7 atom indeed is essential for the mechanism of depurination in the MALDI-TOF process. In conclusion, 7-deazapurine containing nucleic acids show distinctly increased ion-stability and sensitivity under MALDI-TOF conditions and therefore provide for higher mass accuracy and mass resolution.

EXAMPLE 23

Solid State Sequencing and Mass Spectrometer Detection

MATERIALS AND METHODS

Oligonucleotides were purchased from Operon Technologies (Alameda, CA) in an unpurified form. Their sequences are listed in Table III. Sequencing reactions were performed on a solid surface using reagents from the sequencing kit for Sequenase Version 2.0 (Amersham, Arlington Heights, Illinois).

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Sequencing a 39-mer target

Sequencing complex:

5'-TCTGGCCTGGTGCAGGGCCTATTGTAGTTGTGACGTACA-(Aⁿ)₂-3'

5 (DNA11683) (SEQ. ID. No. 13)

3'-TCAACACTGCATGT-5' (PNA16/DNA) (SEQ. ID. No. 14)

In order to perform solid-state DNA sequencing, template strand DNA11683 was 3'-biotinylated by terminal deoxynucleotidyl transferase. A 30 µl reaction, containing 60 pmol of DNA11683, 1.3 nmol of biotin 14-dATP (GIBCO BRL, Grand Island, NY), 30 units of terminal transferase (Amersham, Arlington Heights, Illinois), and 1x reaction buffer (supplied with enzyme), was incubated at 37°C for 1 hour. The reaction was stopped by heat inactivation of the terminal transferase at 70°C for 10 min. The resulting product was desalted by passing through a TE-10 spin column (Clontech). More than one molecules of biotin-14-dATP could be added to the 3'-end of DNA11683. The biotinylated DNA11683 was incubated with 0.3 mg of Dynal streptavidin beads in 30 µl 1x binding and washing buffer at ambient temperature for 30 min. The beads were washed twice with TE and redissolved in 30 µl TE, 10 µl aliquot (containing 0.1 mg of beads) was used for sequencing reactions.

The 0.1 mg beads from previous step were resuspended in a 10 µl volume containing 2 µl of 5x Sequenase buffer (200 mM Tris-HCl, pH 7.5, 100 mM MgCl₂, and 250 mM NaCl) from the Sequenase kit and 5 pmol of corresponding primer PNA16/DNA. The annealing mixture was heated to 70°C and allowed to cool slowly to room temperature over a 20-30 min time period. Then 1 µl 0.1 M dithiothreitol solution, 1 µl Mn buffer (0.15 M sodium isocitrate and 0.1 M MgCl₂), and 2 µl of diluted Sequenase (3.25 units) were added. The reaction mixture was divided into four aliquots of 3 µl each and mixed with termination mixes (each consists of 3 µl of the appropriate termination mix: 32 µM c7dATP, 32 µM dCTP, 32 µM c7dGTP, 32 µM dTTP and 3.2 µM of one of the four ddTNPs, in 50 mM NaCl). The reaction mixtures were incubated at 37°C for 2 min. After the completion of extension, the beads were precipitated and

the supernatant was removed. The beads were washed twice and resuspended in TE and kept at 4 °C.

Sequencing a 78-mer target

5

Sequencing complex:

5'-AAGATCTGACCAGGGATTTCGGTTAGCGTGACTGCTGCTGCTGCTGCTGCTGC
TGGATGATCCGACGCATCAGATCTGG- (A^b)_n-3 (SEQ. ID. NO. 15) (TNR.PLASM2)
3'-CTACTAGGCTGCGTAGTC-5' (CM1) (SEQ. ID. NO. 16)

10 The target TNR.PLASM2 was biotinylated and sequenced using procedures similar to those described in previous section (sequencing a 39-mer target)

Sequencing a 15-mer target with partially duplex probe

Sequencing complex:

5' -T-GATGATCCSACGCAICACAGCTC^{3'} (SEQ. ID. No. 17)
3' -C-CTACTAGGCTGCGTAGTGTGCGAGAACCTTGGCT^{3'} (SEQ. ID. No. 18)

CM1B3B was immobilized on Dynabeads M280 with streptavidin (Dyna-
Norway) by incubating 60 pmol of CM1B3B with 0.3 magnetic beads in 30 μ l 1M NaCl
and TE (1x binding and washing buffer) at room temperature for 30 min. The beads
20 were washed twice with TE and redissolved in 30 μ l TE. 10 or 20 μ l aliquot (containing
0.1 or 0.2 mg of beads respectively) was used for sequencing reactions.

The duplex was formed by annealing corresponding aliquot of beads from previous step with 10 pmol of DF11aSF (or 20 pmol of DF11aSF for 0.2 mg of beads) in a 9 μ l volume containing 2 μ l of 5x Sequenase buffer (200 mM Tris-HCl, pH 7.5, 100 mM MgCl₂, and 250 mM NaCl) from the Sequenase kit. The annealing mixture was heated to 65°C and allowed to cool slowly to 37°C over a 20-30 min time period. The duplex primer was then mixed with 10 pmol of TS10 (20 pmol of TS10 for 0.2 mg of beads) in 1 μ l volume, and the resulting mixture was further incubated at 37°C for 5 min, room temperature for 5-10 min. Then 1 μ l 0.1 M dithiothreitol solution, 1 μ l Mn buffer (0.15 M sodium is citrate and 0.1 M MnCl₂), and 2 μ l of diluted Sequenase (3.25 units) were added. The reaction mixture was divided into four aliquots of 3 μ l each and mixed

with termination mixes (each consists of 4 μ l of the appropriate termination mix: 16 μ M dATP, 16 μ M dCTP, 16 μ M dGTP, 16 μ M dTTP and 1.6 μ M of one of the four ddNTPs, in 50 mM NaCl). The reaction mixtures were incubated at room temperature for 5 min, and 37°C for 5 min. After the completion of extension, the beads were
5 precipitated and the supernatant was removed. The beads were resuspended in 20 μ l TE and kept at 4°C. An aliquot of 2 μ l (out of 20 μ l) from each tube was taken and mixed with 8 μ l of formamide, the resulting samples were denatured at 90-95°C for 5 min and 2 μ l (out of 10 μ l total) was applied to an ALF DNA sequencer (Pharmacia, Piscataway, NJ) using a 10% polyacrylamide gel containing 7 M urea and 0.6x TBE. The remaining
10 aliquot was used for MALDI-TOFMS analysis

MALDI sample preparation and instrumentation

Before MALDI analysis, the sequencing ladder loaded magnetic beads were washed twice using 50 mM ammonium citrate and resuspended in 0.5 μ l pure
15 water. The suspension was then loaded onto the sample target of the mass spectrometer and 0.5 μ l of saturated matrix solution (3-hydroxycolonic acid (HPA) ammonium citrate = 10:1 mole ratio in 50% acetonitrile) was added. The mixture was allowed to dry prior to mass spectrometer analysis

The reflectron TOFMS mass spectrometer (Vision 2000, Finnigan MAT, Bremen, Germany) was used for analysis. 5 kV was applied in the ion source and 20 kV was applied for postacceleration. All spectra were taken in the positive ion mode and a nitrogen laser was used. Normally, each spectrum was averaged for more than 100 shots and a standard 25-point smoothing was applied.

25 RESULTS AND DISCUSSIONS

Conventional solid-state sequencing

In conventional sequencing methods, a primer is directly annealed to the template and then extended and terminated in a Sanger dideoxy sequencing. Normally, a biotinylated primer is used and the sequencing ladders are captured by streptavidin-
30 coated magnetic beads. After washing, the products are eluted from the beads using EDTA and formamide. However, our previous findings indicated that only the annealed

strand of a duplex is desorbed and the immobilized strand remains on the beads (Fang, K. et al., (1995) *Nucleic Acids Research* 23:3126-3131). Therefore, it is advantageous to immobilize the template and anneal the primer. After the sequencing reaction and washing, the beads with the immobilized template and annealed sequencing ladder can be loaded directly onto the mass spectrometer target and mix with matrix. In MALDI, only the annealed sequencing ladder will be desorbed and ionized, and the immobilized template will remain on the target.

A 39-mer template (SEQ. ID. No. 13) was first biotinylated at the 3' end by adding biotin-14-dATP with terminal transferase. More than one biotin-14-dATP molecule could be added by the enzyme. However, since the template was immobilized and remained on the beads during MALDI, the number of biotin-14-dATP would not affect the mass spectra. A 14-mer primer (SEQ. ID. No. 14) was used for the solid-state sequencing. MALDI-TOF mass spectra of the four sequencing ladders are shown in Figure 32, and the expected theoretical values are shown in Table III. The sequencing reaction produced a relatively homogenous ladder, and the full-length sequence was determined easily. One peak around 5150 appeared in all reactions and is not identified. A possible explanation is that a small portion of the template formed some kind of secondary structure, such as a loop, which hindered sequenase extension. Mis-incorporation is of minor importance, since the intensity of these peaks were much lower than that of the sequencing ladders. Although 7-deaza purines were used in the sequencing reaction, which could stabilize the N-glycosidic bond and prevent depurination, minor base losses were still observed since the primer was not substituted by 7-deazapurines. The full length ladder, with a ddA at the 3' end, appeared in the A reaction with an apparent mass of 11899.8. However, a more intense peak of 122 appeared in all four reactions and is likely due to an addition of an extra nucleotide by the Sequenase enzyme.

TABLE III

1.	5'-TCTGGCTGTGTCAGGGGCTATTGTAGTTGACGTACA-(A ⁶)-3'
2.	3'-TCAACACTGCATGT-5'
3.	3'-ATCAAGCTGCATGT-5'
5 4.	3'-CATCAAGCTGCATGT-5'
5.	3'-ACATCAAGCTGCATGT-5'
6.	3'-AACATCAAGCTGCATGT-5'
7.	3'-TAACATCAAGCTGCATGT-5'
8.	3'-ATAACATCAAGCTGCATGT-5'
10 9.	3'-GTAACATCAAGCTGCATGT-5'
10.	3'-GATTAACATCAAGCTGCATGT-5'
11.	3'-GATTAACATCAAGCTGCATGT-5'
12.	3'-GATTAACATCAAGCTGCATGT-5'
13.	3'-CCGGGATTAACATCAAGCTGCATGT-5'
15 14.	3'-TCCGGGATTAACATCAAGCTGCATGT-5'
15.	3'-GTCCGGGATTAACATCAAGCTGCATGT-5'
16.	3'-CGTCCGGGATTAACATCAAGCTGCATGT-5'
17.	3'-ACGTCCGGGATTAACATCAAGCTGCATGT-5'
18.	3'-CAGTCCGGGATTAACATCAAGCTGCATGT-5'
20 19.	3'-CCAGTCCGGGATTAACATCAAGCTGCATGT-5'
20.	3'-ACCAGTCCGGGATTAACATCAAGCTGCATGT-5'
21.	3'-GACAGTCCGGGATTAACATCAAGCTGCATGT-5'
22.	3'-GGACAGTCCGGGATTAACATCAAGCTGCATGT-5'
23.	3'-CGGACAGTCCGGGATTAACATCAAGCTGCATGT-5'
25 24.	3'-CCGACAGTCCGGGATTAACATCAAGCTGCATGT-5'
25.	3'-ACCGACAGTCCGGGATTAACATCAAGCTGCATGT-5'
26.	3'-GACCGACAGTCCGGGATTAACATCAAGCTGCATGT-5'
27.	3'-AGNCCGACAGTCCGGGATTAACATCAAGCTGCATGT-5'

TABLE III CONTINUED

	A-reaction	C-reaction	G-reaction	T-reaction
5	1. 4223.8	4223.8	4223.8	4223.8
	2. 4521.1			
	3. 4809.2	4809.2		
	4. 5122.4			
10	5. 5434.6			
	6. 5737.8			5737.8
	7. 6051.1			
	8. 6379.2		6379.2	
	9. 6704.4		6704.4	
	10. 6995.6			
15	11. 7264.8	6995.6		
	12. 7574.0	7264.8		
	13. 7878.2	7574.0		7878.2
	14. 8207.4		8207.4	
20	15. 8435.6			
	16. 8838.8	8435.6		
	17. 9097.0	8838.8		
	18. 9386.2	9097.0		
	19. 9699.4	9386.2		
25	20. 10027.6		10027.6	
	21. 10355.8		10355.8	
	22. 10644.0			
	23. 10932.2	10644.0		
	24. 11245.4			
30	25. 11574.6		11574.6	
	26. 11885.8			
	27. 12196.9			

- 73 -

The same technique could be used to sequence longer DNA fragments. A 78-mer template containing a CTG repeat (SEQ. ID. No. 15) was 3'-biotinylated by adding biotin-14-dATP with terminal transferase. An 18-mer primer (SEQ. ID. No. 16) was annealed right outside the CTG repeat so that the repeat could be sequenced immediately after primer extension. The four reactions were washed and analyzed by MALDI-TOFMS as usual. An example of the G-reaction is shown in Figure 33 and the expected sequencing ladder is shown in Table IV with theoretical mass values for each ladder component. All sequencing peaks were well resolved except the last component (theoretical value 20577.4) was indistinguishable from the background. Two neighboring sequencing peaks (a 62-mer and a 63-mer) were also separated indicating that such sequencing analysis could be applicable to longer templates. Again, an addition of an extra nucleotide by the Sequenase enzyme was observed in this spectrum. This addition is not template specific and appeared in all four reactions which makes it easy to be identified. Compared to the primer peak, the sequencing peaks were at much lower intensity in the long template case. Further optimization of the sequencing reaction may be required.

[illegible]

TABLE IV (Continued)

TABLE IV (Continued)

	ddAT	ddCTP	ddGTP	ddTTP
	1. 5491.6	5491.6	5491.6	5491.6
	2.	5764.8		
5	3. 6078.0		6407.2	
	4.	6696.4		
	5.			
	6. 7009.6		7338.8	
	7.			
10	8.	7628.0		
	9. 7941.2		8270.4	
	10.			
	11.	8559.6		
	12. 8872.8		9232.0	
15	13.			
	14.	9491.2		
	15. 9804.4		10133.6	
	16.			
	17.	10422.8		
20	18. 10736.0		11065.2	
	19.			
	20.	11354.4		
	21. 11667.6		1196.8	
	22.			
25	23.	12286.0		
	24. 12599.2		12926.4	
	25.			13232.6
	26.			
	27.	13521.8		
30	28. 13835.0			
	29.	14124.2		
	30.		14453.4	
	31.	14742.6		
	32.			15046.8
35	33. 15360.0			
	34. 15673.2			
	35.	15962.4		
	36.	16251.6		
	37.		16580.8	
40	38. 16894.0			
	39. 17207.2			17511.4
	40.			
	41.	17800.6		
	42.	18089.8		
45	43.	18379.0		18683.2
	44.			
	45.		19012.4	
	46.		19341.6	
	47.			19645.8
50	48.	19935.0		
	49. 20248.2		20577.4	
	50.			
	51. 20890.6			21194.8
	52.			
55	53.		21484.0	21788.2
	54.			22092.4
	55.			
60				

Sequencing using duplex DNA probes for capturing and priming

Duplex DNA probes with single-stranded overhang have been demonstrated to be able to capture specific DNA templates and also serve as primers for solid-state sequencing. The scheme is shown in Figure 34. Stacking interactions between a duplex probe and a single-stranded template allow only 5-base overhang to be sufficient for capturing. Based on this format, a 5' fluorescent-labeled 23-mer (5'-GAT GAT CCG ACG CAT CAC AGC TC) (SEQ. ID. No. 19) was annealed to a 3'-biotinylated 18-mer (5'-GTG ATG CGT CGG ATC ATC) (SEQ. ID. No. 20), leaving a 5-base overhang. A 15-mer template (5'-TCG GTT CCA AGA GCT) (SEQ ID. No. 21) was captured by the duplex and sequencing reactions were performed by extension of the 5-base overhang. MALDI-TOF mass spectra of the reactions are shown in Figure 35. All sequencing peaks were resolved although at relatively low intensities. The last peak in each reaction is due to unspecific addition of one nucleotide to the full length extension product by the Sequenase enzyme. For comparison, the same products were run on a conventional DNA sequencer and a stacking fluorogram of the results is shown in Figure 36. As can be seen from the Figure, the mass spectra had the same pattern as the fluorogram with sequencing peaks at much lower intensity compared to the 23-mer primer.

Improvements of MALDI-TOF mass spectrometry as a detection technique

Sample distribution can be made more homogenous and signal intensity could potentially be increased by implementing the picoliter vial technique. In practice, the samples can be loaded on small pits with square openings of 100 μm size. The beads used in the solid-state sequencing is less than 10 μm in diameter, so they should fit well in the microliter vials. Microcrystals of matrix and DNA containing "sweet spots" will be confined in the vial. Since the laser spot size is about 100 μm in diameter, it will cover the entire opening of the vial. Therefore, searching for sweet spots will be unnecessary and high repetition-rate laser (e.g. >10Hz) can be used for acquiring spectra. An earlier report has shown that this device is capable of increasing the detection sensitivity of peptides and proteins by several orders of magnitude compared to conventional MALDI sample preparation technique.

Resolution of MALDI on DNA needs to be further improved in order to extend the sequencing range beyond 100 bases. Currently, using 3-HPA/ammonium citrate as matrix and a reflectron TOF mass spectrometer with 5kV ion source and 20 kV postacceleration, the resolution of the run-through peak in Figure 33 (73-mer) is greater than 200 (FWHM) which is enough for sequence determination in this case. This resolution is also the highest reported for

MALDI desorbed DNA ions above the 70-mer range. Use of the delayed extraction technique may further enhance resolution.

All of the above-cited references and publications are hereby incorporated by reference.

5

EQUIVALENTS

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, numerous equivalents to the specific procedures described herein. Such equivalents are considered to be within the scope of this invention and are covered by the following claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

- 5 (i) APPLICANT: Koster, Hubert
- (ii) TITLE OF INVENTION: DNA SEQUENCING BY MASS SPECTROMETRY
- (iii) NUMBER OF SEQUENCES: 21
- 10 (iv) CORRESPONDENCE ADDRESS:
- (A) ADDRESSEE: Patent Group
Foley, Hoag & Eliot LLP
- (B) STREET: One Post Office Square
- 15 (C) CITY: Boston
- (D) STATE: MA
- (E) COUNTRY: USA
- (F) ZIP: 02109-2170
- 20 (v) COMPUTER READABLE FORM:
- (A) MEDIUM TYPE: Floppy disk
- (B) COMPUTER: IBM PC compatible
- (C) OPERATING SYSTEM: PC-DOS/MS-DOS
- (D) SOFTWARE: ASCII (text)
- 25 (vi) CURRENT APPLICATION DATA:
- (A) APPLICATION NUMBER:
- (B) FILING DATE: 18-MAR-1997
- (C) CLASSIFICATION:
- 30 (vii) PRIOR APPLICATION DATA:
- (A) APPLICATION NUMBER: 08/617,010
- (B) FILING DATE: 18-MAR-1996
- 35 (viii) PRIOR APPLICATION DATA:
- (A) APPLICATION NUMBER: 08/178,215
- (B) FILING DATE: 06-JAN-1994
- (C) CLASSIFICATION:
- 40 (ix) ATTORNEY/AGENT INFORMATION:
- (A) NAME: Arnold, Beth E.
- (B) REGISTRATION NUMBER: 35,430
- (C) REFERENCE/DOCKET NUMBER: SQA-3.25.27

(x) TELECOMMUNICATION INFORMATION:

(A) TELEPHONE: (617) 832-1294

(B) TELEFAX: (617) 832-7000

5

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

10 (A) LENGTH: 14 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

15 (ii) MOLECULE TYPE: other nucleic acid

(iii) HYPOTHETICAL: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

20

CATGCCATGG CATG

14

(2) INFORMATION FOR SEQ ID NO:2:

25 (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

30

(ii) MOLECULE TYPE: other nucleic acid

(iii) HYPOTHETICAL: YES

35

(x) SEQUENCE DESCRIPTION: SEQ ID NO:2:

AAATTGTGCA CATCCTGCAG C

21

40

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

- 81 -

- (A) LENGTH: 50 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

5

(ii) MOLECULE TYPE: other nucleic acid

(iii) HYPOTHETICAL: YES

10

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

TAACGGTCAT TACGGCCATT GACTGTAGGA CCTGCATTAC ATGACTAGCT

50

15

(2) INFORMATION FOR SEQ ID NO:4:

(1) SEQUENCE CHARACTERISTICS:

20

- (A) LENGTH: 17 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

25

(ii) MOLECULE TYPE: other nucleic acid

(iii) HYPOTHETICAL: YES

30

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

TAAAACGACG GGGCAGXG

37

35

(2) INFORMATION FOR SEQ ID NO:5

(1) SEQUENCE CHARACTERISTICS:

40

- (A) LENGTH: 17 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(iii) HYPOTHETICAL: YES

5

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

XAAAACGACC GGCCASXG

17

10

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

15

(A) LENGTH: 18 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

20

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

25

GTCACCCTCG ACCTGCAG

18

(2) INFORMATION FOR SEQ ID NO:7:

30

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 19 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

35

(ii) MOLECULE TYPE: cDNA

40

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

TTGTAAAACG ACGGCCAGT

19

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:

- 5 (A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

10

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

15 CTTCCACCGC GATGTTGA

18

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:

- 20 (A) LENGTH: 17 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

25 (ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

30

CAGGAAACAG CTATGAC

17

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:

- 35 (A) LENGTH: 17 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

40

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

GTAAACGAC GGCCAGT

17

5 (2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:

- 10 (A) LENGTH: 19 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

15

(ix) FEATURE:

- 20 (A) NAME/KEY: misc_feature
(B) LOCATION: 1..19
(C) OTHER INFORMATION: /note= "All lowercase letters
represent RiboG"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

25 GTCACCCTCG ACCTGCAgC

19

(2) INFORMATION FOR SEQ ID NO:12:

(1) SEQUENCE CHARACTERISTICS:

- 30 (A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

35 (ii) MOLECULE TYPE: cDNA

(ix) FEATURE:

- 40 (A) NAME/KEY: misc_feature
(B) LOCATION: 1..20
(C) OTHER INFORMATION: /note= "All lowercase letters
represent RiboG"

(x1) SEQUENCE DESCRIPTION: SEQ ID NO:12:

GTGTGTAAGG GAGGGCCAGT

20

5 (2) INFORMATION FOR SEQ ID NO:13:

(1) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 39 base pairs

(B) TYPE: nucleic acid

10 (C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

15

(x1) SEQUENCE DESCRIPTION: SEQ ID NO:13:

TCTGGCCTGG TGCAAGGCCT ATTGTAGTTG TGACGTACA

39

20 (2) INFORMATION FOR SEQ ID NO:14:

(1) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 14 base pairs

(B) TYPE: nucleic acid

25 (C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

30

(x1) SEQUENCE DESCRIPTION: SEQ ID NO:14:

TCAACACTGC ATGT

14

35 (2) INFORMATION FOR SEQ ID NO:15:

(1) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 78 base pairs

(B) TYPE: nucleic acid

40 (C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

5 AAGATCTGAC CAGGGATTGG GTTAGCGTGA CTGCTGCTGC TGCTGCTGCT GCTGGATGAT 65
CCGACGCATC AGATCTGG 78

(2) INFORMATION FOR SEQ ID NO:16:

10

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
15 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

20

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

CTACTAGGCT GCGTAGTC 18

25 (2) INFORMATION FOR SEQ ID NO:17:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
30 (C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

35

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

GATGATCCGA CGCATCACAG CTC 23

40 (2) INFORMATION FOR SEQ ID NO:18:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 33 base pairs

(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

5 (ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

10 CTACTAGGCT GCCTAGTGTC GAGAACCTTG GCT

33

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:

15 (A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

20 (ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

25

GATGATCCGA CGCATCACAG CTC

23

(2) INFORMATION FOR SEQ ID NO:20:

30 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

35

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

40

GTGATGCGTC GGATCATC

18

(2) INFORMATION FOR SEQ ID NO:21:

(1) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 15 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

5 (D) TOPOLOGY: linear

(2) MOLECULE TYPE: cDNA

10

(3) SEQUENCE DESCRIPTION: SEQ ID NO:21:

TCGGTTCCAA GAGGT

35

CLAIMS

- 5 I. A method for determining the sequence of a nucleic acid, comprising the steps of:
- a) generating at least two base-specifically terminated nucleic acid fragments containing modified purine nucleotides that are relatively resistant to fragmentation during mass spectrometry;
- 10 b) determining the molecular weight value of each base-specifically terminated fragment by mass spectrometry, wherein the molecular weight values of at least two base-specifically terminated fragments are determined concurrently; and
- 15 c) determining the sequence of the nucleic acid by aligning the base-specifically terminated nucleic acid fragments according to molecular weight.
2. The method according to claim 1 wherein the nucleic acid fragments are purified before the step of determining the molecular weight values by mass spectrometry.
- 20 3. The method according to claim 2 wherein the nucleic acid fragments are purified, comprising the steps of:
- a) reversibly immobilizing the nucleic acid fragments on a solid support; and
- b) washing out all remaining reactants and by-products.
- 30 4. The method according to claim 3, further comprising the step of removing the nucleic acid fragments from the solid support.

5. The method of claim 1, wherein the fragments contain deazapurine moieties.
6. The method of claim 1, wherein the deaza purine moieties are selected from the group consisting of: C¹-deazaadenine, C²-deazaguanine, 7-deazainosine triphosphate, C⁸-deazaadenine, C⁹-deazaguanine and C⁹-deazainosine triphosphate.
7. The method of claim 1, wherein at least about 50% of the purine nucleotides are modified within the nucleotide fragment.
8. A process of claim 1 wherein the mass spectrometer is selected from the group consisting of: Matrix-Assisted Laser Desorption/Ionization Time-of-Flight (MALDI-TOF), Electrospray (ES), Ion Cyclotron Resonance (ICR), and Fourier Transform and combinations thereof.
9. The method according to claim 1, wherein more than one species of nucleic acid are concurrently sequenced by multiplex mass spectrometric nucleic acid sequencing employing nucleic acid primers, chain-elongating nucleotides, and chain-terminating nucleotides, wherein one of the sets of base-specifically terminated fragments is unmodified and the other sets of base-specifically terminated nucleic acid fragments are mass modified, and each of the sets of base-specifically terminated nucleic acid fragments has a sufficient mass difference to be distinguished from the others by mass spectrometry.
10. The method according to claim 9, wherein at least one of the sets of mass-modified base-specifically terminated fragments is modified with a mass-modifying functionality at a heterocyclic base of at least one nucleotide.
11. The method according to claim 10, wherein the heterocyclic base-modified nucleotide is selected from the group consisting of a cytosine nucleotide modified at C-5, a thymine nucleotide modified at C-5, a thymine nucleotide

modified at the C-5 methyl group, a uracil nucleotide modified at C-5, an adenine nucleotide modified at C-8, an adenine nucleotide modified at C-7, a c⁷-deazaadenine modified at C-8, a c⁷-deazaadenine modified at C-7, a guanine nucleotide modified at C-8, a guanine nucleotide modified at C-7, a c⁷-deazaguanine modified at C-8, a c⁷-deazaguanine modified at C-7, a hypoxanthine modified at C-8, a c⁷-deazahypoxanthine modified at C-7, and a c⁷-deazahypoxanthine modified at C-8.

12. The method according to claim 9, wherein at least one of the sets of mass-modified base-specifically terminated nucleic acid fragments is modified with a mass-modifying functionality attached to one or more phosphate moieties of the internucleotidic linkages of the fragments

13. The method according to claim 9, wherein at least one of the sets of mass-modified base-specifically terminated nucleic acid fragments is modified with a mass-modifying functionality attached to one or more sugar moieties of nucleotides within the set of mass modified base-specifically terminated fragments at at least one sugar position selected from the group consisting of a C-2' position, an external C- 3' position, and an external C-5' position.

14. The method according to claim 9, wherein at least one of the sets of mass-modified base-specifically terminated nucleic acid fragments is modified with a mass-modifying functionality (M) attached to the sugar moiety of a 5'-terminal nucleotide and wherein the mass-modifying function (M) is the linking functionality (L).

15. The method according to claim 9, wherein a mass-modifying functionality (M) is attached to a set of base-specifically terminated nucleic acid fragments subsequent to generating the base-specifically terminated nucleic acid fragments and prior to determining the molecular weight values for the nested fragments by mass spectrometry.

16. The method according to claim 15, wherein the base-specifically terminated nucleic acid fragments are generated using at least one reagent selected from the group consisting of a nucleic acid primer, a chain-elongating nucleotide, a chain-terminating nucleotide and a tag probe which has been modified with a precursor of the mass-modifying functionality, M; and a subsequent step comprises
5 modifying the precursor of the mass-modifying functionality to generate the mass-modifying functionality, M, prior to mass spectrometric analysis.

17. The method according to claim 9, wherein mass differentiation of the tag probes is achieved by changing the nucleotide composition of at least one of the
10 tag probes and complementary tag sequence in the species of nucleic acid.

18. The method according to claim 9, wherein the tag probes are covalently bound to the corresponding complementary tag sequence prior to mass
15 spectrometric analysis.

19. The method according to claim 18, wherein binding between the tag probes and the corresponding complementary tag sequences is achieved photochemically via photoactivatable groups.
20

20 A method of sequencing a nucleic acid, comprising the steps of:

a) reversibly linking an oligonucleotide primer to a solid support;

b) generating at least two base-specifically terminated nucleic acid
25 fragments containing nucleotides that are relatively resistant to fragmentation during mass spectrometry,

c) determining the molecular weight value of each nested fragment in each of the four sets of base-specifically terminated fragments of the nucleic acid by matrix assisted laser desorption/ionization mass spectrometry
30 wherein the molecular weight values of at least two base-specifically terminated

fragments are determined concurrently and wherein the nested fragments are cleaved from the solid support by a laser during mass spectrometry; and

d) determining the nucleotide sequence by aligning the base specifically terminated fragments according to molecular weight.

21. The method according to claim 20 wherein the nucleic acid fragments are purified before the step of determining the molecular weight values by mass spectrometry.

22. The method according to claim 21 wherein the nucleic acid fragments are purified, comprising the steps of:

a) reversibly immobilizing the nucleic acid fragments on a solid support; and

b) washing out all remaining reactants and by-products.

23. The method according to claim 22, further comprising the step of removing the nucleic acid fragments from the solid support.

24. The method of claim 20, wherein the fragments contain deazapurine moieties.

25. The method of claim 20, wherein the deaza purine moieties are selected from the group consisting of: C⁷-deazaadenine, C⁷-deazaguanine, 7-deazainosine triphosphate, C⁹-deazaadenine, C⁹-deazaguanine and C⁹-deazainosine triphosphate.

26. The method of claim 20, wherein at least about 50% of the purine nucleotides are modified within the nucleotide fragment.

27. A process of claim 20 wherein the mass spectrometer is selected from the group consisting of: Matrix-Assisted Laser Desorption/Ionization Time-of-Flight (MALDI-TOF), Electrospray (ES), Ion Cyclotron Resonance (ICR), and Fourier Transform and combinations thereof.

28. The method according to claim 20, wherein more than one species of nucleic acid are concurrently sequenced by multiplex mass spectrometric nucleic acid sequencing employing nucleic acid primers, chain-elongating nucleotides, and chain-terminating nucleotides, wherein one of the sets of base-specifically terminated fragments is unmodified and the other sets of base-specifically terminated nucleic acid fragments are mass modified, and each of the sets of base-specifically terminated nucleic acid fragments has a sufficient mass difference to be distinguished from the others by mass spectrometry.

29. The method according to claim 28, wherein at least one of the sets of mass-modified base-specifically terminated fragments is modified with a mass-modifying functionality (M) at a heterocyclic base of at least one nucleotide.

30. The method according to claim 29, wherein the heterocyclic base-modified nucleotide is selected from the group consisting of a cytosine nucleotide modified at C-5, a thymine nucleotide modified at C-5, a thymine nucleotide modified at the C-5 methyl group, a uracil nucleotide modified at C-5, an adenine nucleotide modified at C-8, an adenine nucleotide modified at C-7, a c^7 -deazaadenine modified at C-8, a c^7 -deazaadenine modified at C-7, a guanine nucleotide modified at C-8, a guanine nucleotide modified at C-7, a c^7 -deazaguanine modified at C-8, a c^7 -deazaguanine modified at C-7, a hypoxanthine modified at C-8, a c^7 -deazahypoxanthine modified at C-7, and a c^7 -deazahypoxanthine modified at C-8.

31. The method according to claim 28, wherein at least one of the sets of mass-modified base-specifically terminated nucleic acid fragments is modified with a

mass-modifying functionality (M) attached to one or more phosphate moieties of the internucleotidic linkages of the fragments.

5 32. The method according to claim 28, wherein at least one of the sets of mass-modified base-specifically terminated nucleic acid fragments is modified with a mass-modifying functionality (M) attached to one or more sugar moieties of nucleotides within the set of mass modified base-specifically terminated fragments at at least one sugar position selected from the group consisting of a C-2' position, an external C- 3' position, and an external C-5' position.

10

33 The method according to claim 28, wherein at least one of the sets of mass-modified base-specifically terminated nucleic acid fragments is modified with a mass-modifying functionality (M) attached to the sugar moiety of a 5'-terminal nucleotide and wherein the mass-modifying function (M) is the linking
15 functionality (I.).

34. The method according to claim 28, wherein a mass-modifying functionality (M) is attached to a set of base-specifically terminated nucleic acid fragments subsequent to generating the base-specifically terminated nucleic acid fragments and prior to determining the molecular weight values for the nested fragments by
20 mass spectrometry.

35. The method according to claim 34, wherein the base-specifically terminated nucleic acid fragments are generated using at least one reagent
25 selected from the group consisting of a nucleic acid primer, a chain-elongating nucleotide, a chain-terminating nucleotide and a tag probe which has been modified with a precursor of the mass-modifying functionality, M; and a subsequent step comprises modifying the precursor of the mass-modifying functionality, M, to generate the mass-modifying functionality, M, prior to mass
30 spectrometric analysis.

36. The method according to claim 28, wherein mass differentiation of the tag probes is achieved by changing the nucleotide composition of at least one of the tag probes and complementary tag sequence in the species of nucleic acid.

5 37. The method according to claim 28, wherein the tag probes are covalently bound to the corresponding complementary tag sequence prior to mass spectrometric analysis.

10 38. The method according to claim 37, wherein binding between the tag probes and the corresponding complementary tag sequences is achieved photochemically via photoactivatable groups.

39. A method of multiplex analysis of nucleic acid sequences, comprising the steps of:

- 15 a) reversibly linking a nucleic acid primer to a solid support;
- b) generating at least two conditioned, base-specifically terminated nucleic acid fragments containing modified purine nucleotides that are relatively resistant to fragmentation during mass spectrometry;
- 20 c) determining the molecular weight value of each fragment by matrix assisted laser desorption/ionization mass spectrometry wherein the molecular weight values of at least two base-specifically terminated fragments are determined concurrently and wherein the fragments are cleaved from the solid support by a laser during mass spectrometry; and
- 25 d) determining the nucleotide sequence by aligning the fragments according to molecular weight; wherein at least one reagent selected from a group consisting of a nucleic acid primer, a chain-elongating nucleotide, and a chain-terminating nucleotide which has been mass-modified; wherein each set of base-specifically
- 30 terminated fragments has a sufficient mass difference from the other sets of base-specifically terminated fragments so as to be unique; and wherein the molecular

weight values of the nested fragments of two or more sets of unseparated base-specifically terminated fragments are determined concurrently.

5 40. The method according to claim 39, wherein the reversible linkage is a photocleavable bond.

41. The method according to claim 39 wherein the base-specifically terminated fragments are cleaved from the solid support prior to mass spectrometry.

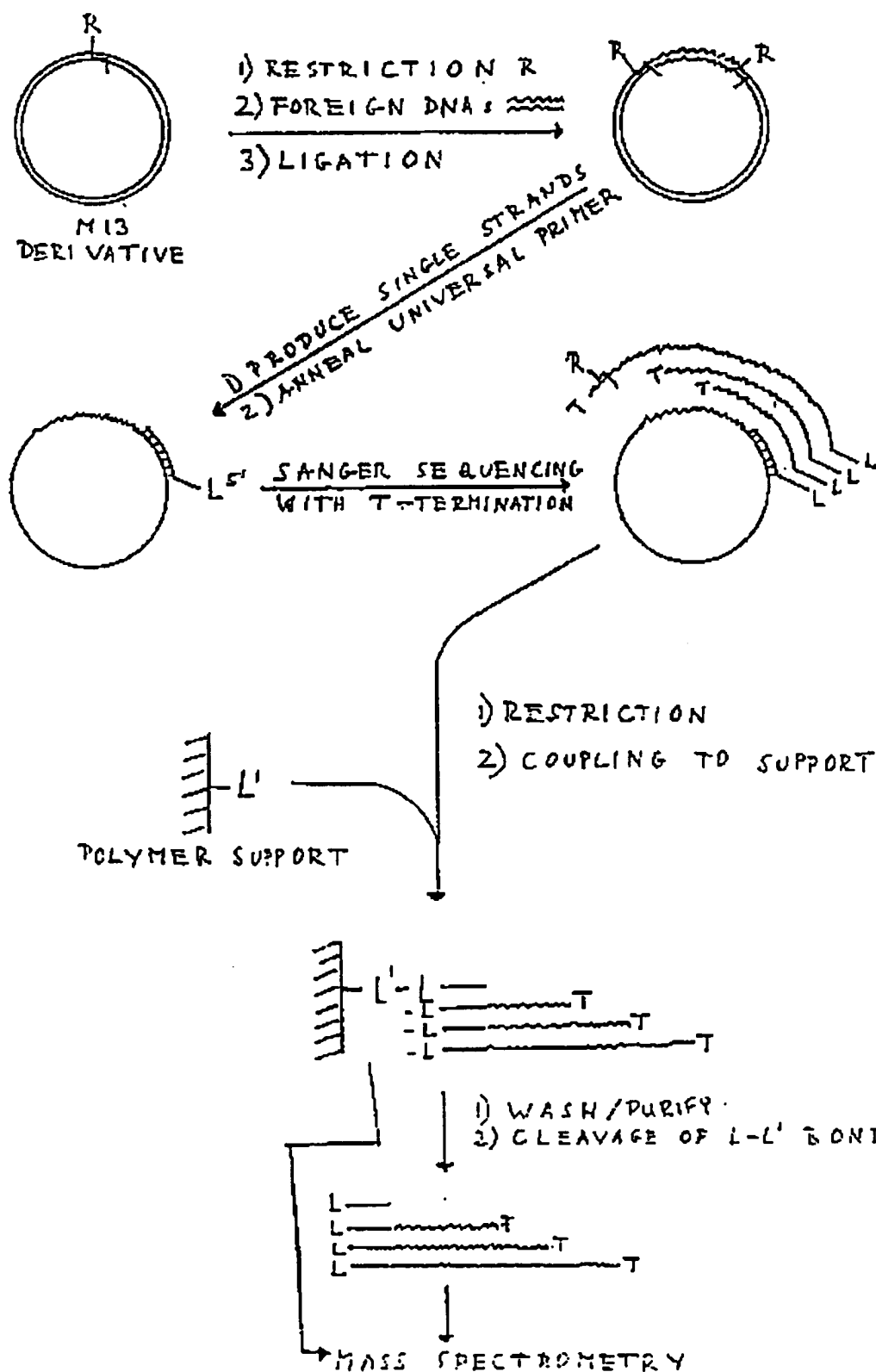


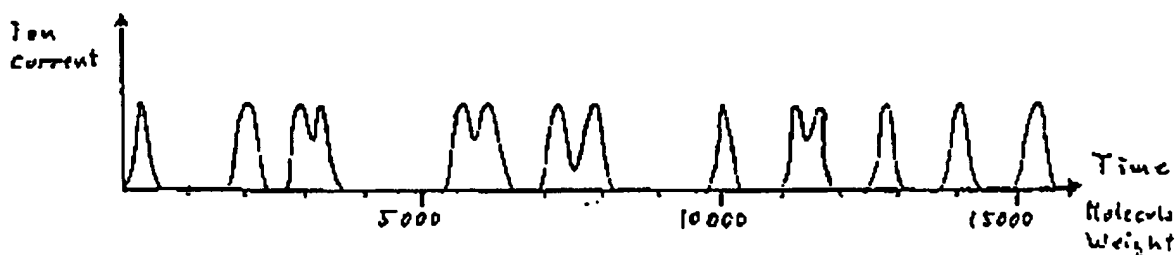
FIGURE 1

FIGURE 7

Figure 2A: Sanger DNA Sequencing Reaction Products with
ddT Termination of Hypothetical DNA (50-mer).

5'-dTAAACGGTCAATTACGGCCATTGACTGTAGGACCTGCATTACATGACTAGCT	
5'-dT	226.23
5'-dTAAACGGT	2104.45
5'-dTAAACGGTCAT	3011.04
5'-dTAAACGGTCATT	3315.24
5'-dTAAACGGTCATTACGGCCAT	5771.82
5'-dTAAACGGTCATTACGGCCATT	6076.02
5'-dTAAACGGTCATTACGGCCATTGACT	7311.82
5'-dTAAACGGTCATTACGGCCATTGACTGT	7945.22
5'-dTAAACGGTCATTACGGCCATTGACTGTAGGACCT	10112.63
5'-dTAAACGGTCATTACGGCCATTGACTGTAGGACCTGCAT	11348.43
5'-dTAAACGGTCATTACGGCCATTGACTGTAGGACCTGCATT	11652.62
5'-dTAAACGGTCATTACGGCCATTGACTGTAGGACCTGCATTACAT	12872.42
5'-dTAAACGGTCATTACGGCCATTGACTGTAGGACCTGCATTACATGACT	14108.22
5'-dTAAACGGTCATTACGGCCATTGACTGTAGGACCTGCATTACATGACTAGCT	15344.02

Figure 2B: Idealized MALDI-TOF Mass Spectrum Showing the
"ion current vs. time/molecular weight".

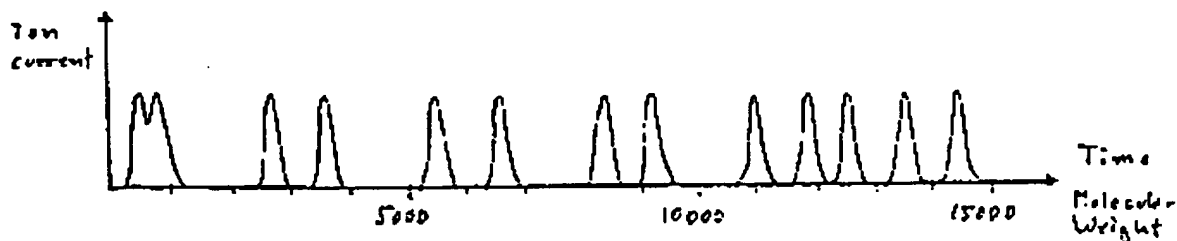


1	5'-dTp	molecular weight increments used:	305.20412
1	3'-ddT		225.22422
12	dTp		304.19618
13	dAp		313.20954
12	dCp		289.16454
11	dGp		329.20694
50	Nucleotide B		

Figure 3A: Sanger DNA Sequencing Reaction Products with
dda Termination of Hypothetical DNA (50-mer).

5'-dTACGGTCATTACGGCCATTGACTGTAGGACCTGCATTACATGACTAGCT	
5'-dT	530.43
5'-dTAA	843.64
5'-dTACGGTCA	2697.83
5'-dTACGGTCATTA	3619.43
5'-dTACGGTCATTACGGCCA	5458.61
5'-dTACGGTCATTACGGCCATTGA	6709.42
5'-dTACGGTCATTACGGCCATTGACTGTA	8249.42
5'-dTACGGTCATTACGGCCATTGACTGTAGGA	9221.05
5'-dTACGGTCATTACGGCCATTGACTGTAGGACCTGCA	11035.22
5'-dTACGGTCATTACGGCCATTGACTGTAGGACCTGCATTA	11956.82
5'-dTACGGTCATTACGGCCATTGACTGTAGGACCTGCATTACA	12559.21
5'-dTACGGTCATTACGGCCATTGACTGTAGGACCTGCATTACATGA	13505.83
5'-dTACGGTCATTACGGCCATTGACTGTAGGACCTGCATTACATGACTA	14412.42

Figure 3B: Idealized MALDI-TOF Mass Spectrum Showing the
"ion current vs. time/molecular weight".



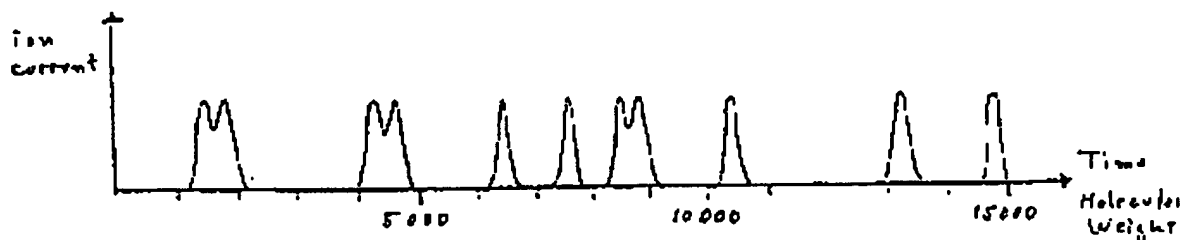
1	5'-dTp	molecular weight increments used:	305.20412
1	3'-ddA		225.22422
13	dTp		304.19616
12	dAp		313.20954
12	dCp		289.18454
12	dGp		329.20894
53	Nucleotides		

FIGURE 3

Figure 4A: Sanger DNA Sequencing Reaction Products with
ddG Termination of Hypothetical DNA (50-mer).

5'-dTAACGGTCATTACGGGCATTGACTGTAGGACCTGCATTACATGACTAGCT	
5'-dTAACG	1471.05
5'-dTAACGG	1800.25
5'-dTAACGGTCATTACG	4246.84
5'-dTAACGGTCATTACGG	4576.05
5'-dTAACGGTCATTACGGGCCATTG	6405.23
5'-dTAACGGTCATTACGGGCCATTGACTG	7641.03
5'-dTAACGGTCATTACGGGCCATTGACTGTAG	8587.64
5'-dTAACGGTCATTACGGGCCATTGACTGTAGG	8916.85
5'-dTAACGGTCATTACGGGCCATTGACTGTAGGACCTG	10441.84
5'-dTAACGGTCATTACGGGCCATTGACTGTAGGACCTGCATTACATG	13201.63
5'-dTAACGGTCATTACGGGCCATTGACTGTAGGACCTGCATTACATGACTAG	14750.64

Figure 4B: Idealized MALDI-TOF Mass Spectrum Showing the
"ion current vs. time/molecular weight".



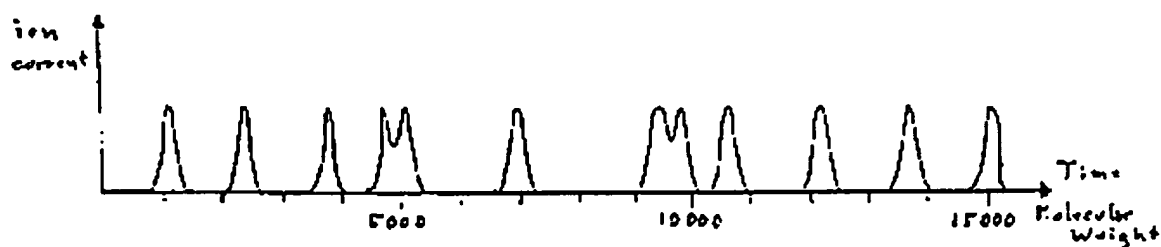
1	5'-dTp	molecular weight increments used:	305.20412
2	3'-ddG		250.23698
12	dTp		304.17618
13	dAp		313.25954
12	dCp		289.18454
11	dGp		329.20394
50	Nucleotides		

FIGURE 4

Figure 5A: Sanger DNA Sequencing Reaction Products with
ddC Termination of Hypothetical DNA (50-mer).

5'-dTAAACGGTCATTACGGCCATTGACTGTAGGACCTGCATTACATGACTAGCT	
5'-dTAAAC	1141.84
5'-dTAAACGGTC	2393.63
5'-dTAAACGGTCATTAC	3917.63
5'-dTAAACGGTCATTACGGC	4865.23
5'-dTAAACGGTCATTACGGCC	5154.42
5'-dTAAACGGTCATTACGGCCATTGAC	7007.62
5'-dTAAACGGTCATTACGGCCATTGACTGTAGGAC	9519.25
5'-dTAAACGGTCATTACGGCCATTGACTGTAGGACC	9808.43
5'-dTAAACGGTCATTACGGCCATTGACTGTAGGACCTGC	10731.02
5'-dTAAACGGTCATTACGGCCATTGACTGTAGGACCTGCATTAC	12255.62
5'-dTAAACGGTCATTACGGCCATTGACTGTAGGACCTGCATTACATGAC	13804.62
5'-dTAAACGGTCATTACGGCCATTGACTGTAGGACCTGCATTACATGACTAGC	15039.62

Figure 5B: Idealized MALDI-TOF Mass Spectrum Showing the
"ion current vs. time/molecular weight".



1	5'-dTp	molecular weight increments used:	305.20412
1	3'-ddC		220.21250
12	dTp		304.19610
23	dAp		313.20954
32	dCp		289.18454
11	dGp		329.20 94
50	Nucleotides		

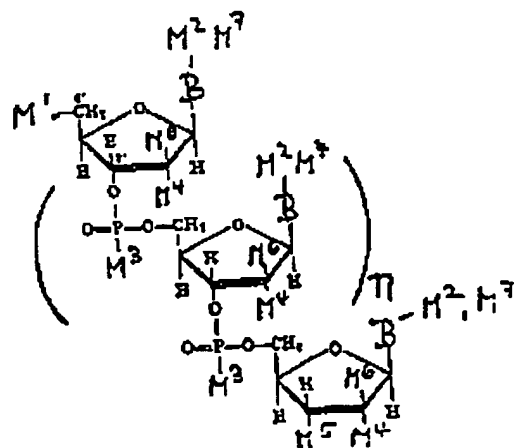
FIGURE 5

FIGURE 6

Figure 6: Series of Molecular Weights of the Hypothetical DNA (50-mer) after Sanger Sequencing and Termination with either ddTPT, ddATP, ddGTP or ddCTP respectively.

	ddT	ddA	ddG	ddC
T	226.23			
A		530.43		
C		843.64		
G			1471.05	1141.84
G			1800.25	
T	2104.45			
C				2393.63
A		2697.83		
T	3011.04			
T	3315.24			
A		3619.43		
C				3917.63
G			4246.84	
G			4576.05	
C				4865.23
C				5154.42
A		5458.61		
T	5771.82			
T	6076.02			
G			6405.23	
A		6709.42		
C				7007.62
T	7311.82			
G			7641.03	
T	7945.22			
A		8249.42		
G			8587.64	
G			8916.05	
A		9221.05		
C				9519.25
C				9808.43
T	10112.63			
G			10461.84	
C				10731.02
A		11035.22		
T	11348.43			
T	11652.62			
A		11956.82		
C				12255.02
A		12559.21		
T	12872.42			
G			13201.63	
A		13505.83		
C				13804.02
T	14108.22			
A		14412.42		
C			14750.64	
C				15039.82
T	15344.02			

FIGURE 7


 $n = 1 - 50$

$M = H, OH, XR,$
Halogen: N_3

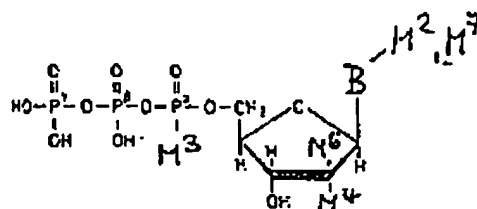
	M ¹	M ²	M ³	M ⁵
Type Ia (base modified DNA)	OH	XR/Hal	OH	H
Type Ib (base modified RNA)	OH	XR/Hal	OH	OH
Type IIA (5'-modified DNA)	XR/Hal	H	OH	H
Type IIB (5'-modified RNA)	XR/Hal	H	OH	OH
Type III (3'-modified)	OH	H	OH	XR/Hal
Type IVa (P-modified DNA)	OH	H	XR	H
Type IVb (P-modified RNA)	OH	H	XR	OH

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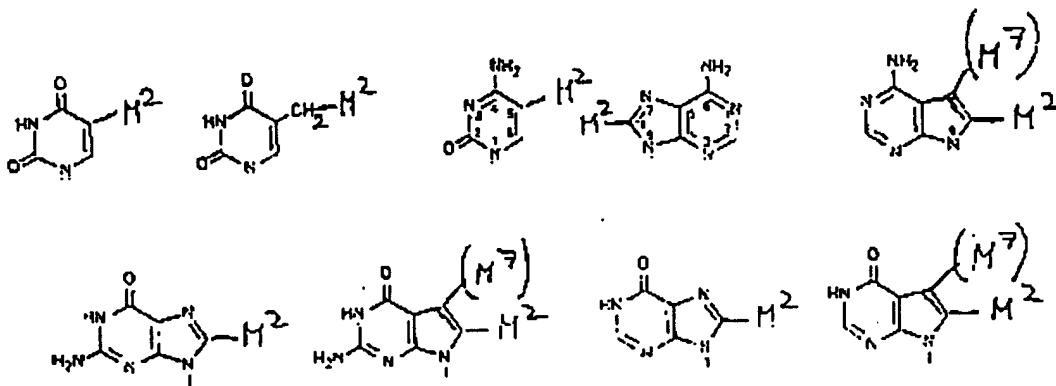
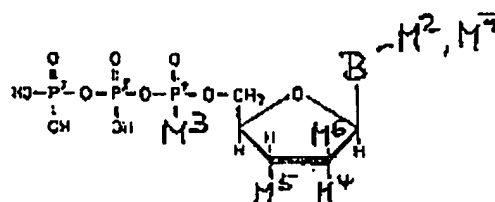
FIGURE 8

Nucleoside Triphosphate Elongators:

A



Nucleoside Triphosphate Terminators:

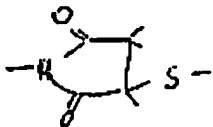


B

	H ²	H ³	H ⁴	H ⁵
Type A (DNA-Termination)	XR	OH	H	H
Type B (DNA-Termination)	H	OH	H	XR
Type C (DNA-Termination)	H	XR	H	H
Type D (RNA-Termination)	XR	OH	OH	H
Type E (RNA-Termination)	H	OH	OH	XR
Type F (RNA-Termination)	H	XR	OH	H

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FIGURE 9

X	R
-O-	-(CH ₂ CH ₂ O) _m -CH ₂ CH ₂ -OH
	or -(CH ₂ CH ₂ O) _m -CH ₂ CH ₂ -O-Alkyl
-O-C(=O)-(CH ₂) _r -C(=O)-O-	-(CH ₂ CH ₂ O) _m -CH ₂ CH ₂ -OH
	or -(CH ₂ CH ₂ O) _m -CH ₂ CH ₂ -O-Alkyl
-NH-C(=O)- / -C(=O)-NH-	-(CH ₂ CH ₂ O) _m -CH ₂ CH ₂ -OH
	or -(CH ₂ CH ₂ O) _m -CH ₂ CH ₂ -O-Alkyl
-NH-C(=O)-(CH ₂) _r -C(=O)-O-	-(CH ₂ CH ₂ O) _m -CH ₂ CH ₂ -OH
	or -(CH ₂ CH ₂ O) _m -CH ₂ CH ₂ -O-Alkyl
-NH-C(=S)-NH-	-(CH ₂ CH ₂ O) _m -CH ₂ CH ₂ -OH
	or -(CH ₂ CH ₂ O) _m -CH ₂ CH ₂ -O-Alkyl
-O-P(=O)(O-Alkyl)-	-(CH ₂ CH ₂ O) _m -CH ₂ CH ₂ -OH
	or -(CH ₂ CH ₂ O) _m -CH ₂ CH ₂ -O-Alkyl
-O-SO ₂ -O-	-(CH ₂ CH ₂ O) _m -CH ₂ CH ₂ -OH
	or -(CH ₂ CH ₂ O) _m -CH ₂ CH ₂ -O-Alkyl
-O-C(=O)-CH ₂ -S-	-(CH ₂ CH ₂ O) _m -CH ₂ CH ₂ -OH
	or -(CH ₂ CH ₂ O) _m -CH ₂ CH ₂ -O-Alkyl
	-(CH ₂ CH ₂ O) _m -CH ₂ CH ₂ -OH
	or -(CH ₂ CH ₂ O) _m -CH ₂ CH ₂ -O-Alkyl
-S-	-(CH ₂ CH ₂ O) _m -CH ₂ CH ₂ -OH
	or -(CH ₂ CH ₂ O) _m -CH ₂ CH ₂ -O-Alkyl
-NH-	-(CH ₂ CH ₂ O) _m -CH ₂ CH ₂ -CH
	or -(CH ₂ CH ₂ O) _m -CH ₂ CH ₂ -O-Alkyl

m = 0, 1 - 200

r = 1 - 20

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FIGURE 10

Mass modifying functionality R (X similar to FIGURE 9):

-R

Alkyl: $-(CH_2)_x-CH_3$ e.g. $-CH_3$, $-C_2H_5$,
and branched e.g. $-CH(CH_3)_2$

$ICH_2(CH_2)_x-O-H$

2,3-Epoxy-1-propanol

$-(CH_2)_m-CH_2-O-H$

$-(CH_2)_m-CH_2-O-Alkyl$

$-(CH_2CH_2NH)_m-CH_2CH_2-NH_2$

$-[NH-(CH_2)_x-NH-C(=O)-(CH_2)_x-C(=O)]_m-NH-(CH_2)_x-NH-C(=O)-(CH_2)_x-C(=O)-OH$

$-[NH(CH_2)_x-C(=O)]_m-NH-(CH_2)_x-C(=O)-OH$

$-[NH-CHY-C(=O)]_m-NH-CHY-C(=O)-OH$

$-[O-(CH_2)_x-C(=O)]_m-O-(CH_2)_x-C(=O)-OH$

-S-

-Si(Alkyl)₃

-Halogen

-N₃

$-CH_2F$, $-CHF_2$, $-CF_3$

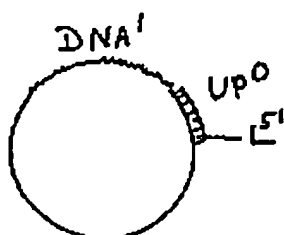
$m = 0, 1 - 200$

$x = 1 - 20$

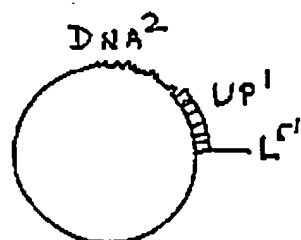
11/43

FIGURE 11

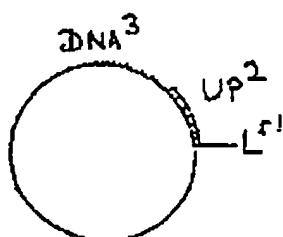
e.g. for dT-Termination:



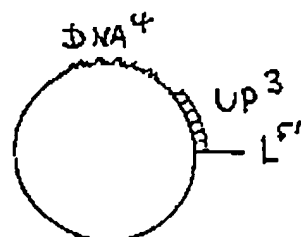
ddTTP⁰
dTTP⁰
dATP⁰
dGTP⁰
dCTP⁰



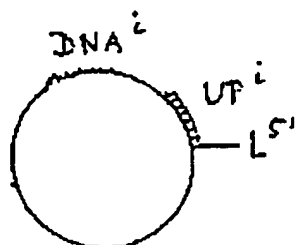
ddTTP⁰
dTTP⁰
dATP⁰
dGTP⁰
dCTP⁰



ddTTP⁰
dTTP⁰
dATP⁰
dGTP⁰
dCTP⁰



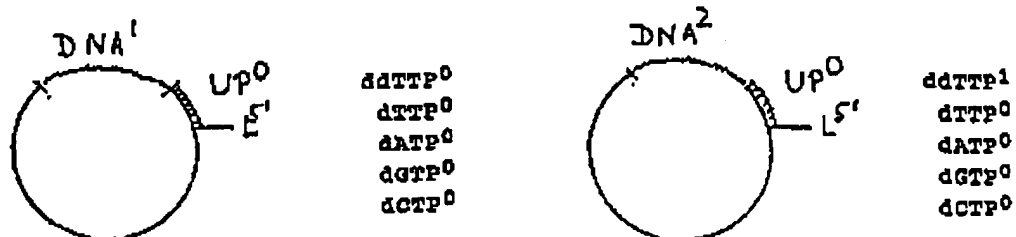
ddTTP⁰
dTTP⁰
dATP⁰
dGTP⁰
dCTP⁰



ddTTP⁰
dTTP⁰
dATP⁰
dGTP⁰
dCTP⁰

FIGURE 12

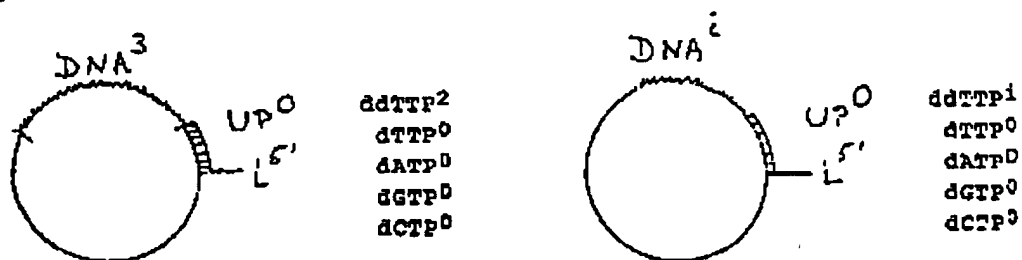
e.g. for dT-Termination:



for signal amplification elongation with:

<p>dTTP⁰ dATP⁰ dGTP⁰ dCTP⁰</p>	<p>ddTTP¹ dATP⁰ dGTP⁰ dCTP⁰</p>
--	---

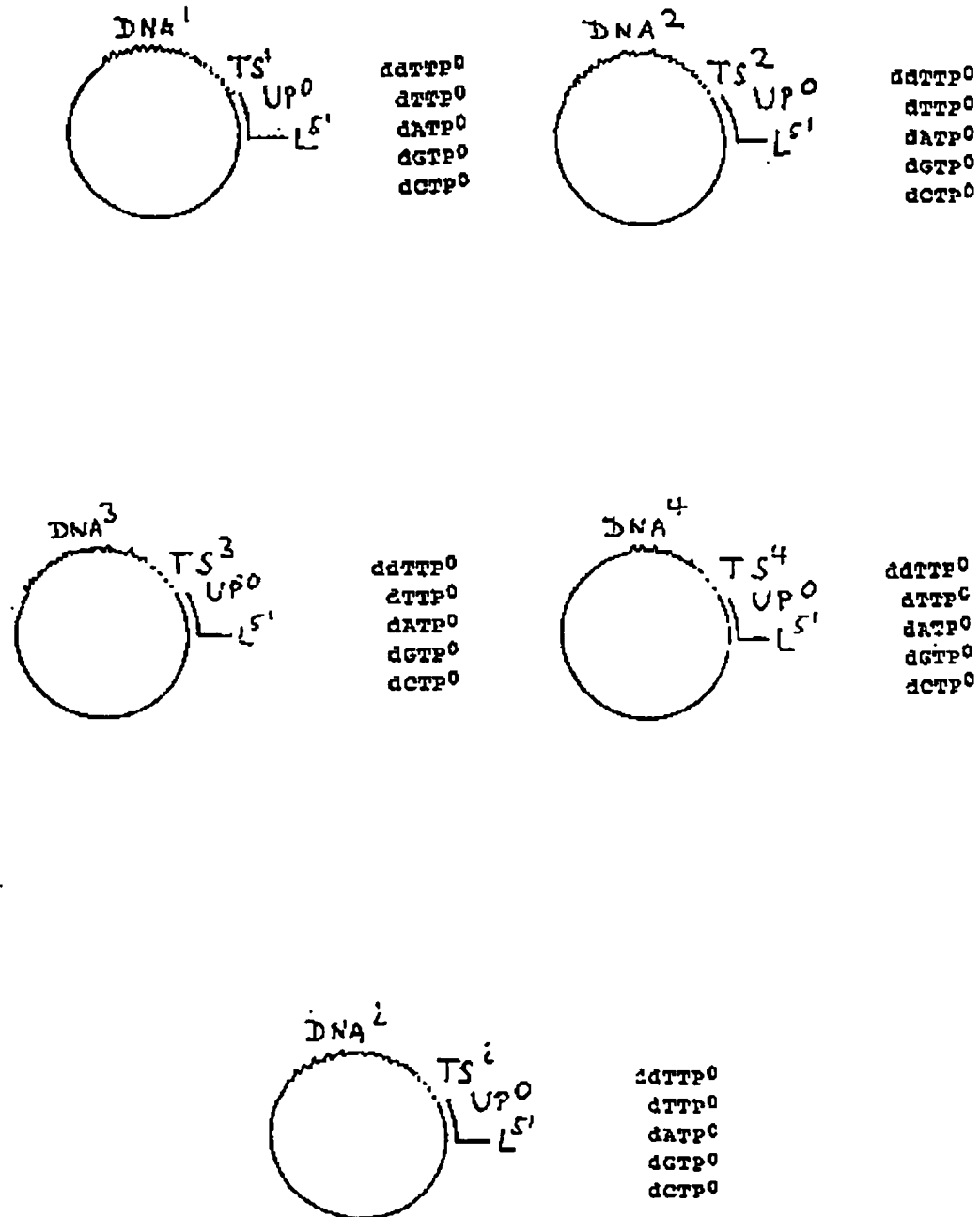
e.g. for dT-Termination:



for signal amplification elongation with:

<p>dTTP² dATP⁰ dGTP⁰ dCTP⁰</p>	<p>ddTTP¹ dATP⁰ dGTP⁰ dCTP⁰</p>
--	---

e.g. for dT-Termination:



TS = TAG SEQUENCE

FIGURE 13.

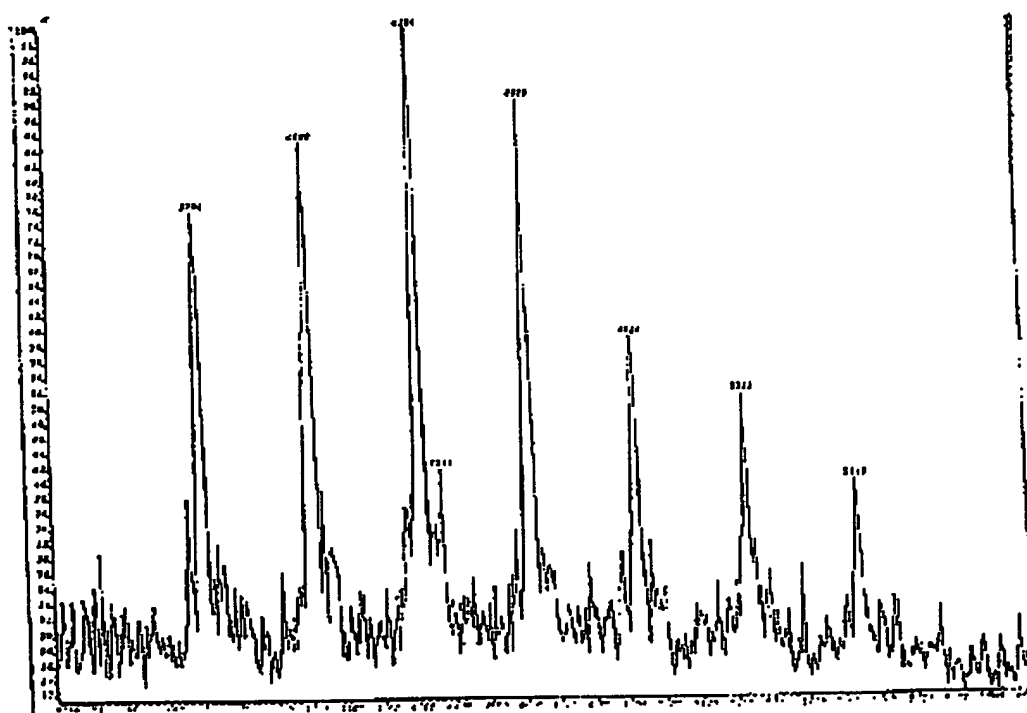


FIGURE 14

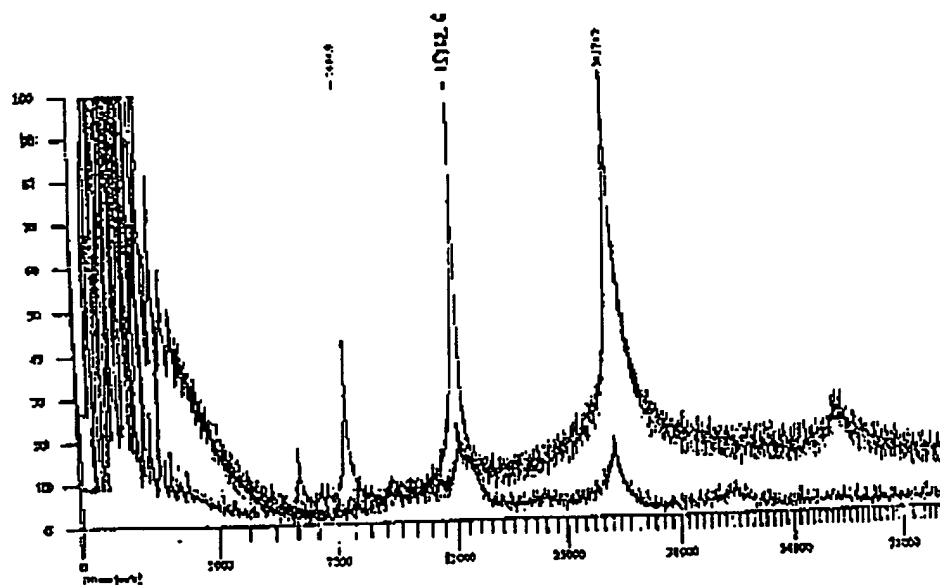


FIGURE 15

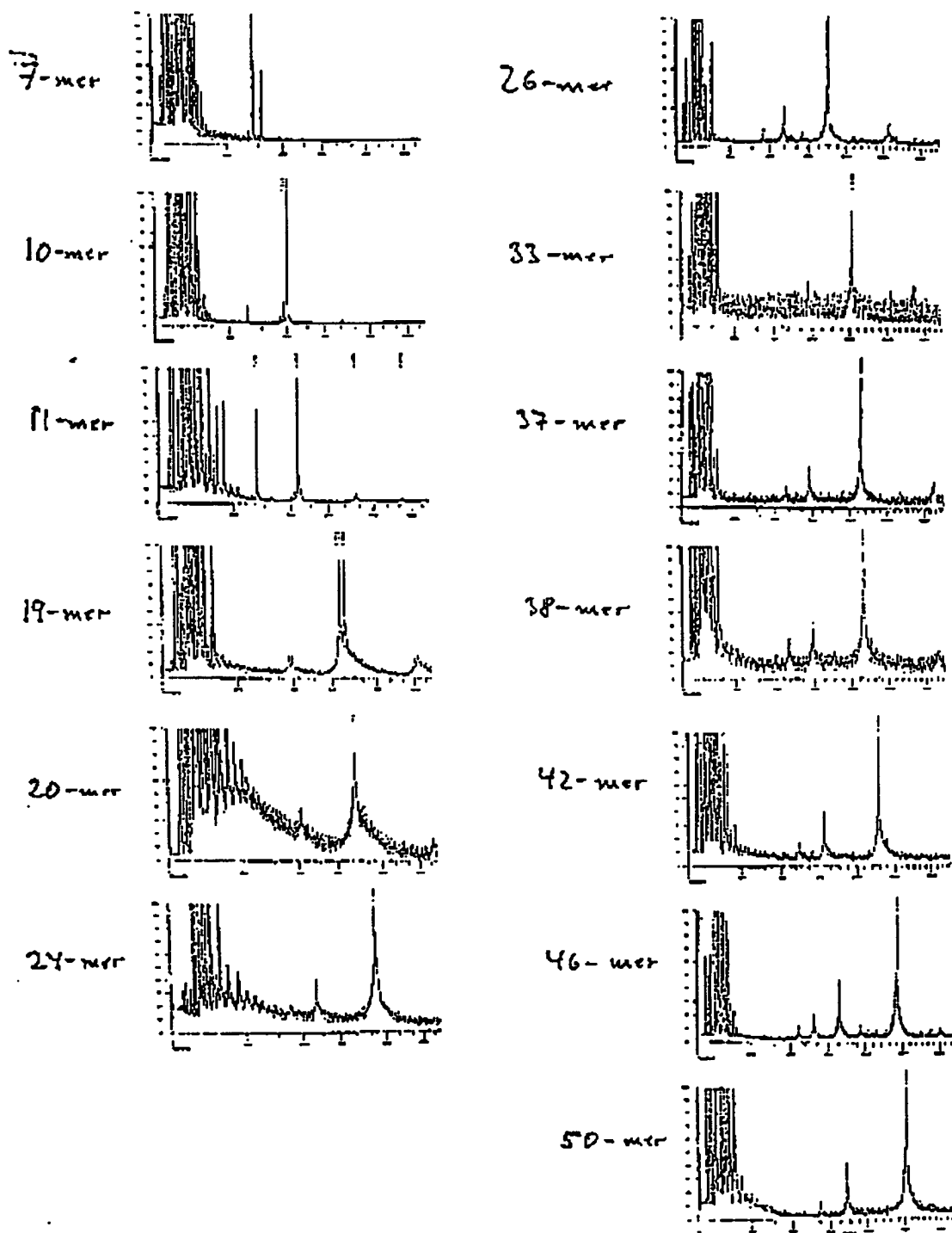


FIGURE 16

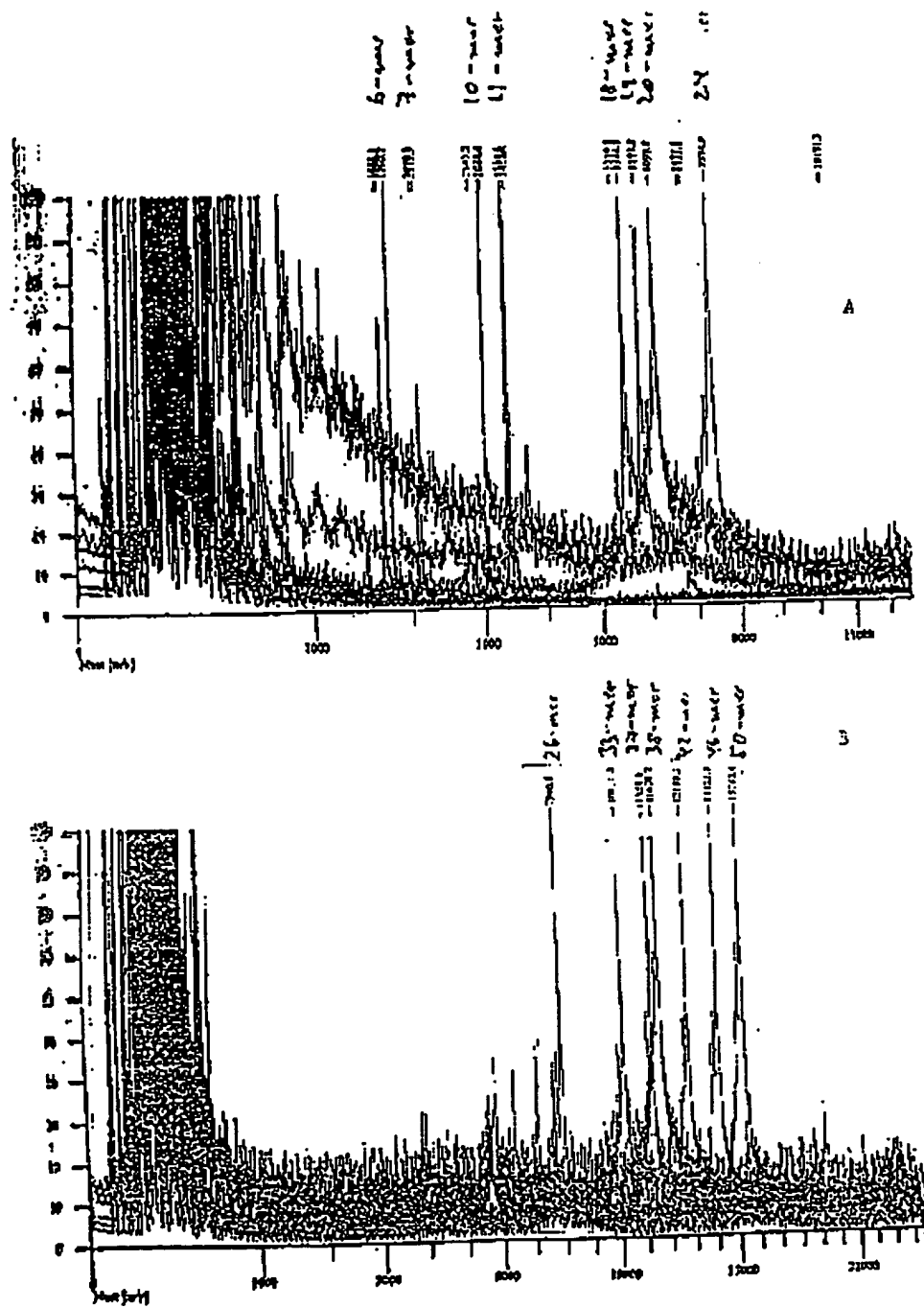


FIGURE 17

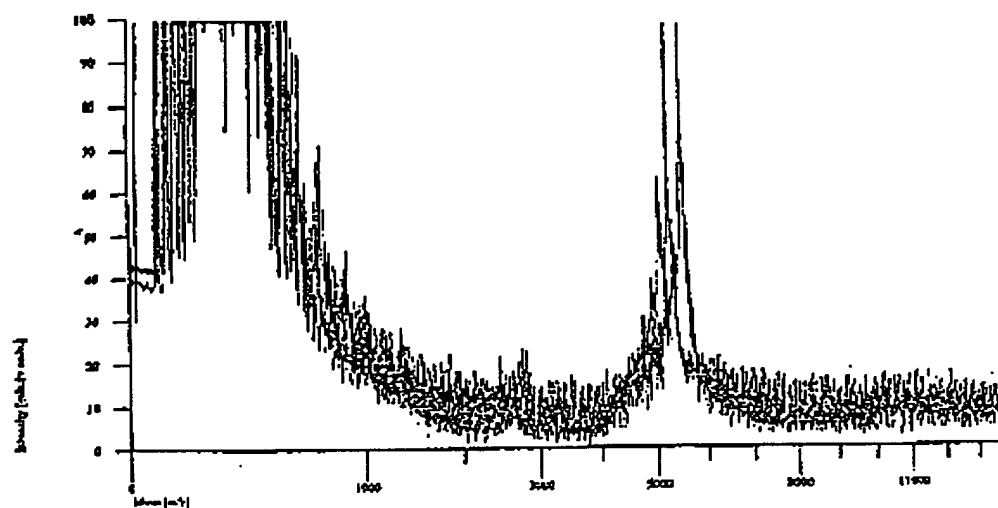
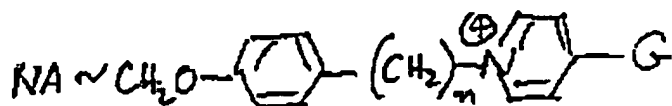
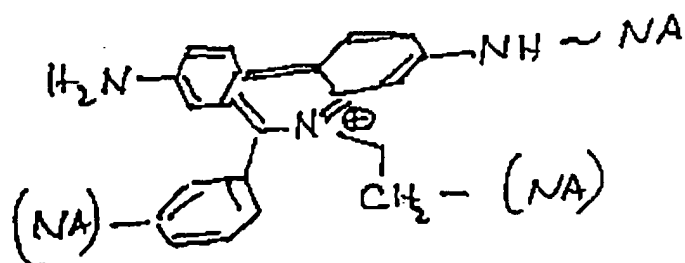
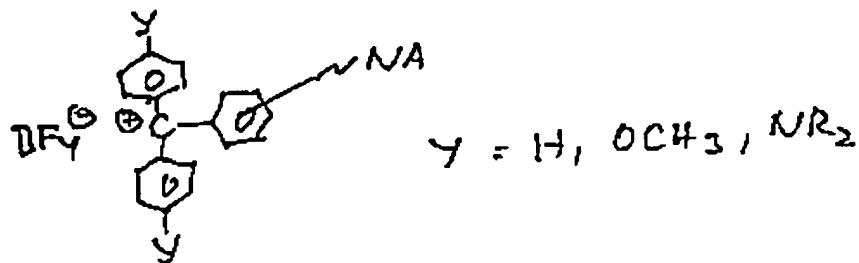
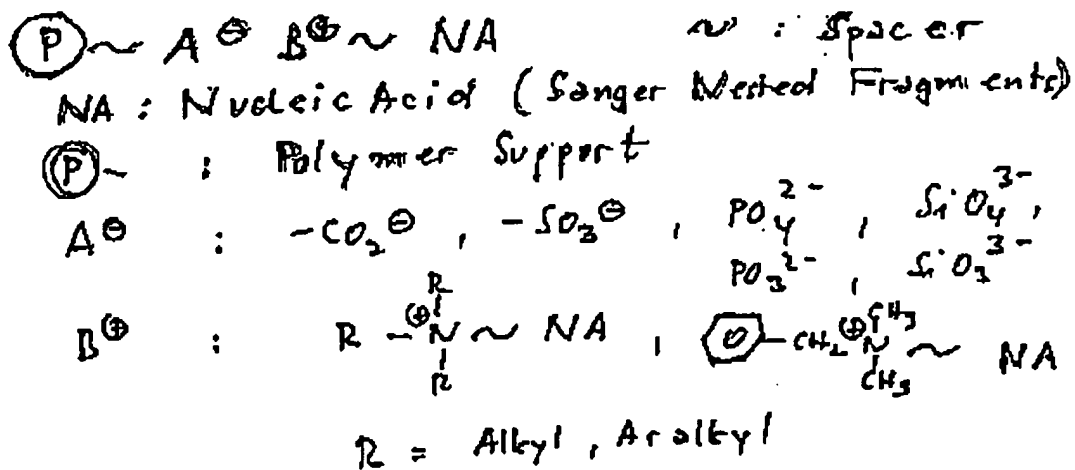


FIGURE 18



$$n = 1 - 10$$

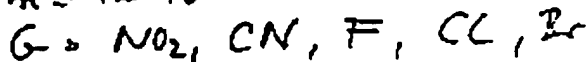


FIGURE 19


 \sim : Spacer


: Polymer Support



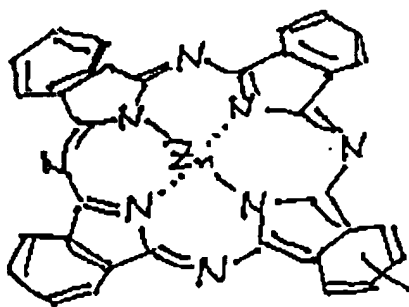
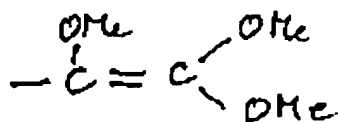
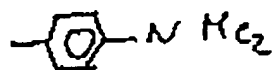
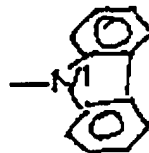
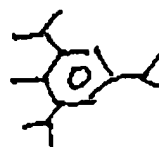
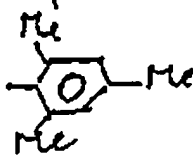
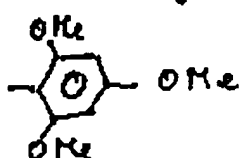
: Nucleic Acid (Sanger Marked Fragments)



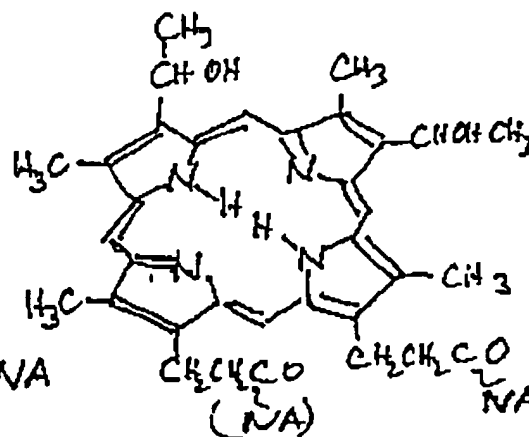
: Charge Transfer Acceptor



: Charge Transfer Donator

D:


Zinc Phthalocyanine



Hematoporphyrin

FIGURE 20

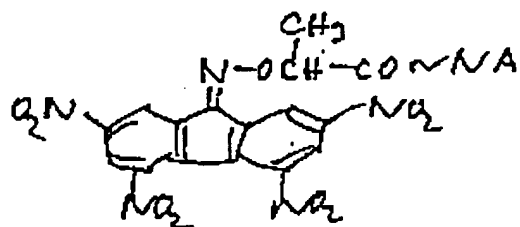
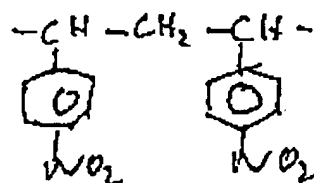
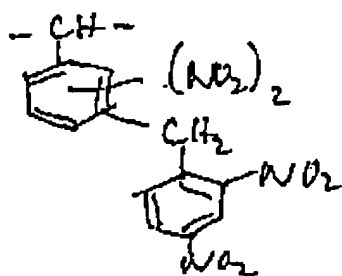
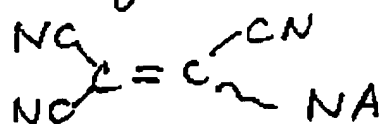
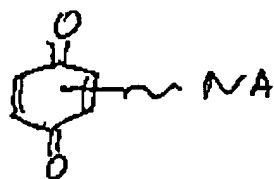
A:

FIGURE 20, CONT

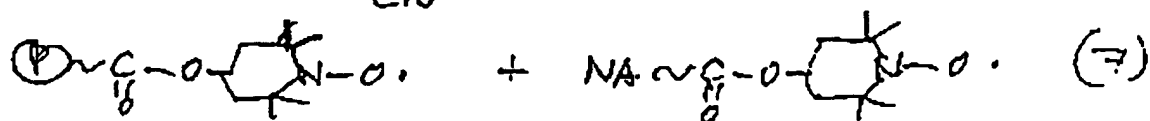
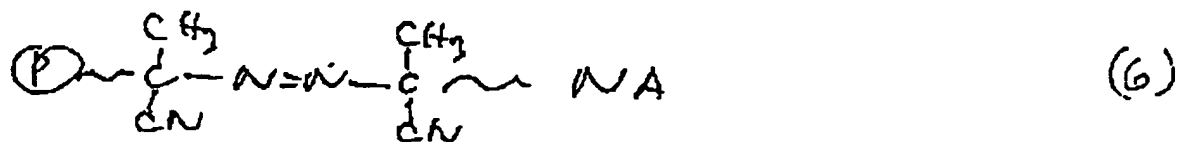
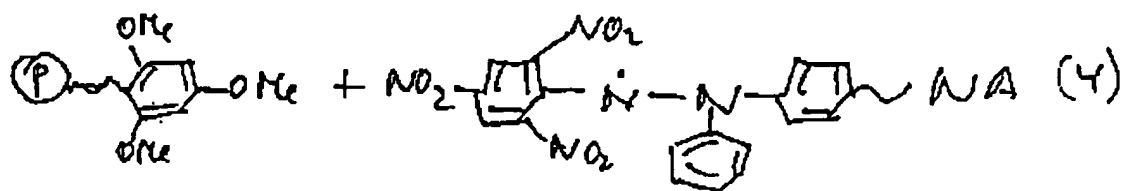
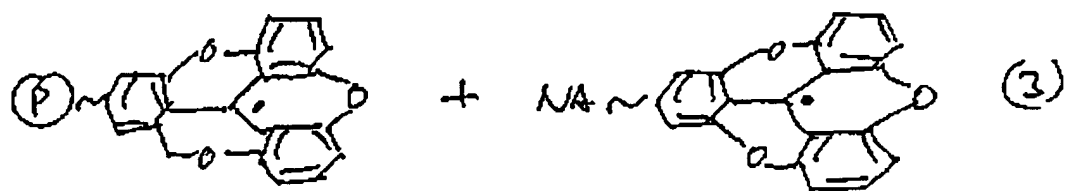
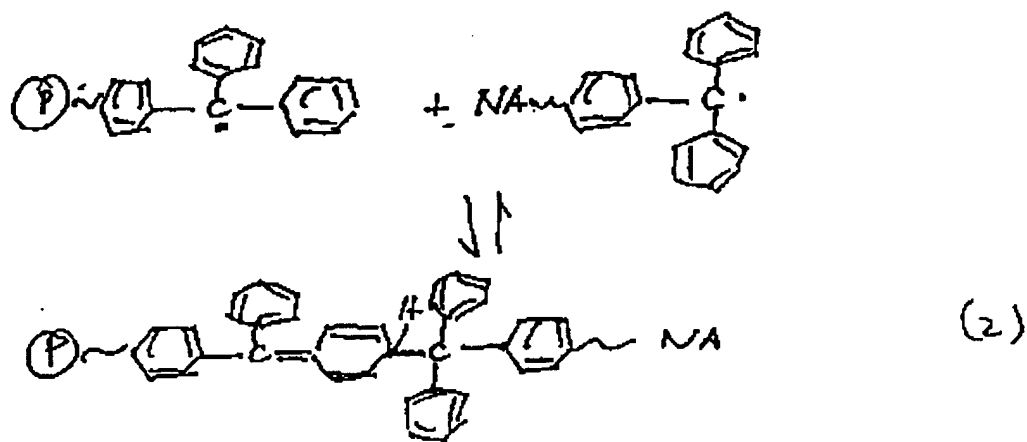
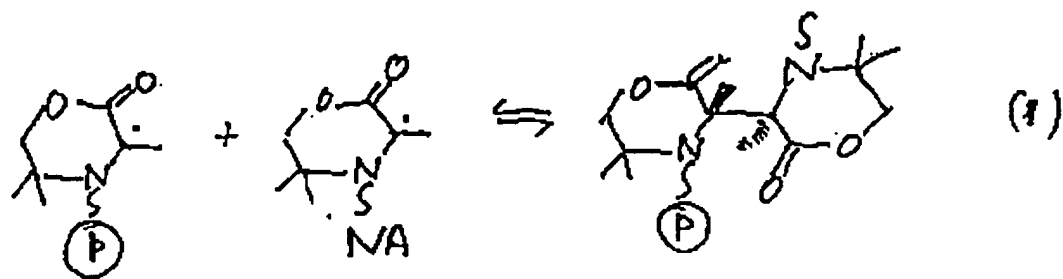
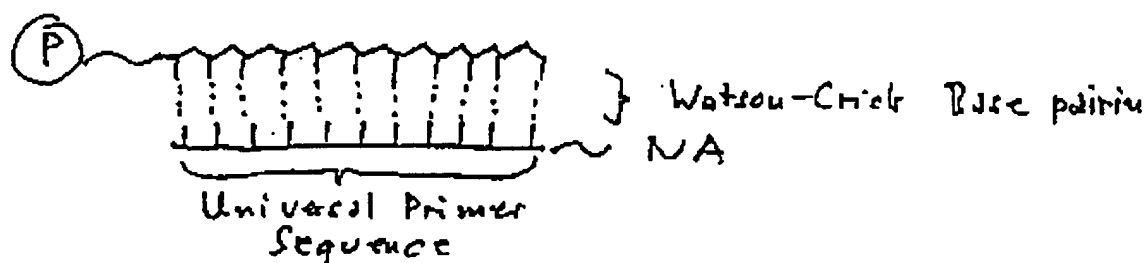


FIGURE 21



NA : Nucleic Acid (Nested Sugar Fragments)

(P) : Polymer Support


 : Oligo ribo-/-deoxyribo-
 nucleotide or
 Oligonucleotide mimetic
 " " " "

FIGURE 22



P : POLYMER SUPPORT

\sim : SPACER

h.v : PHOTOCLEAVABLE BOND

NA : NUCLEIC ACID
(NESTED SANGER FRAGMENTS)

FIGURE 23

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FIGURE 26

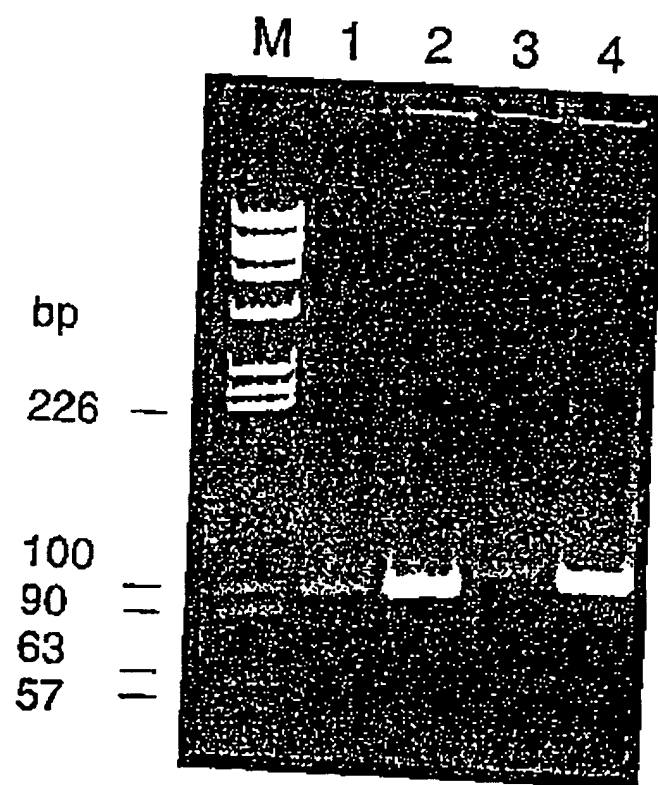
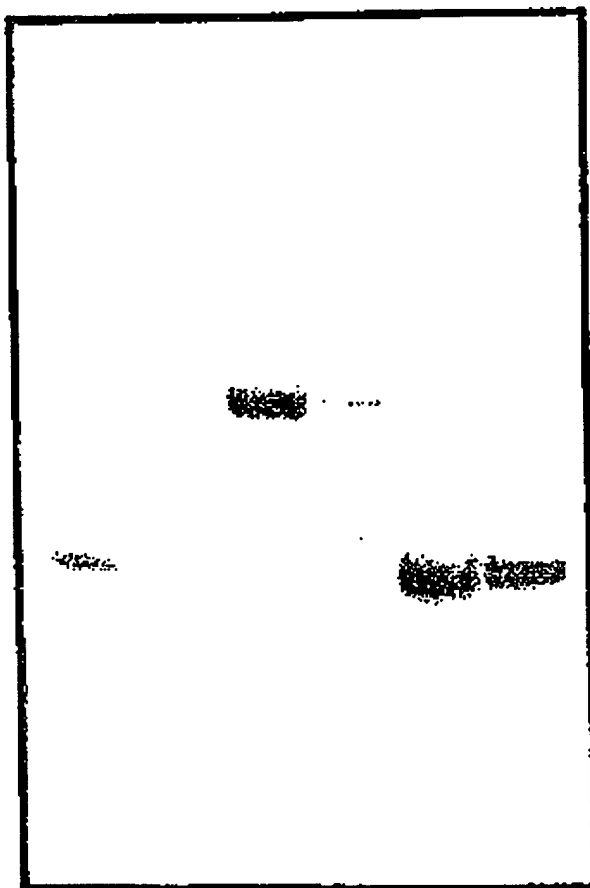


FIGURE 27

1 2 3 4 5 6



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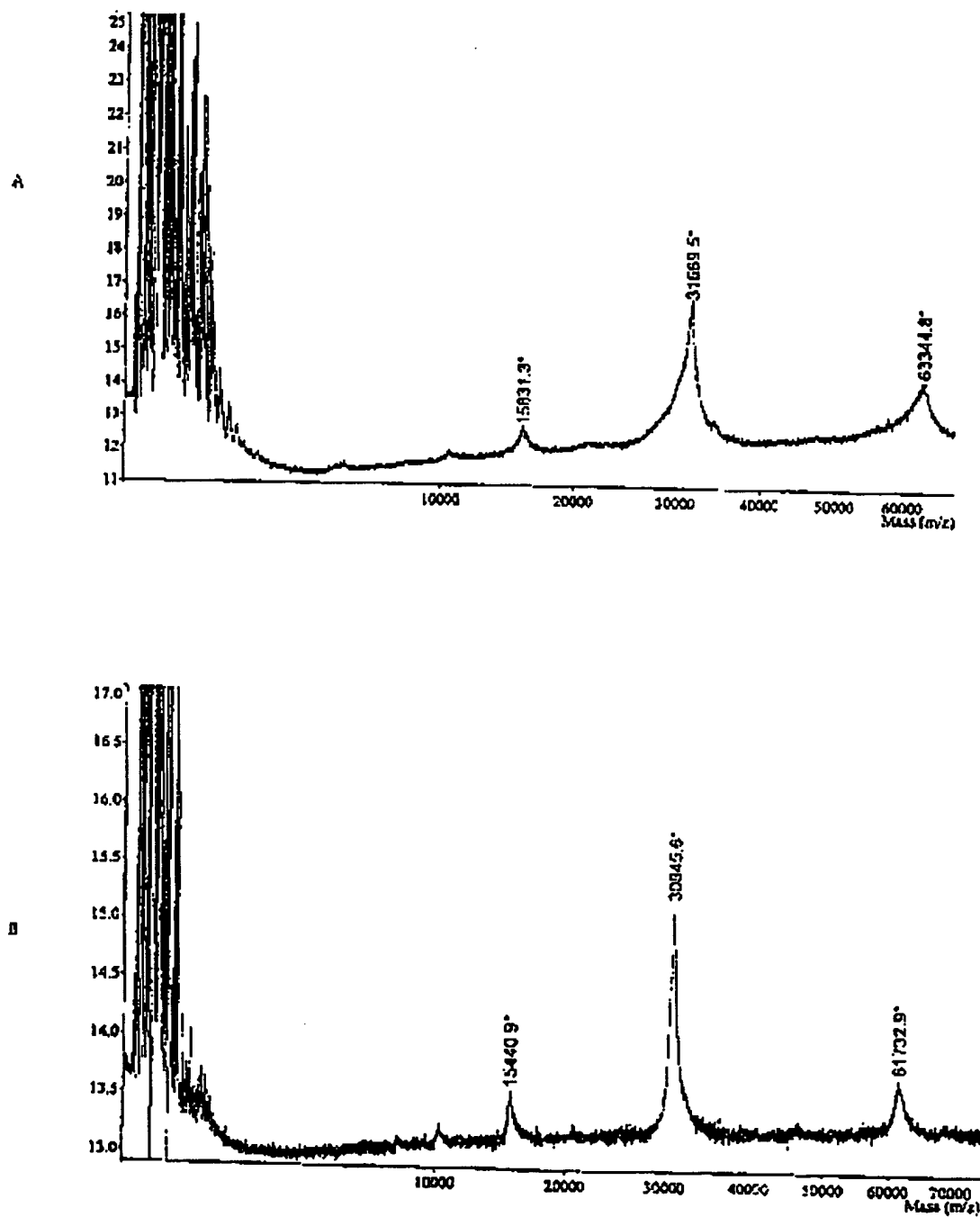


FIGURE 28

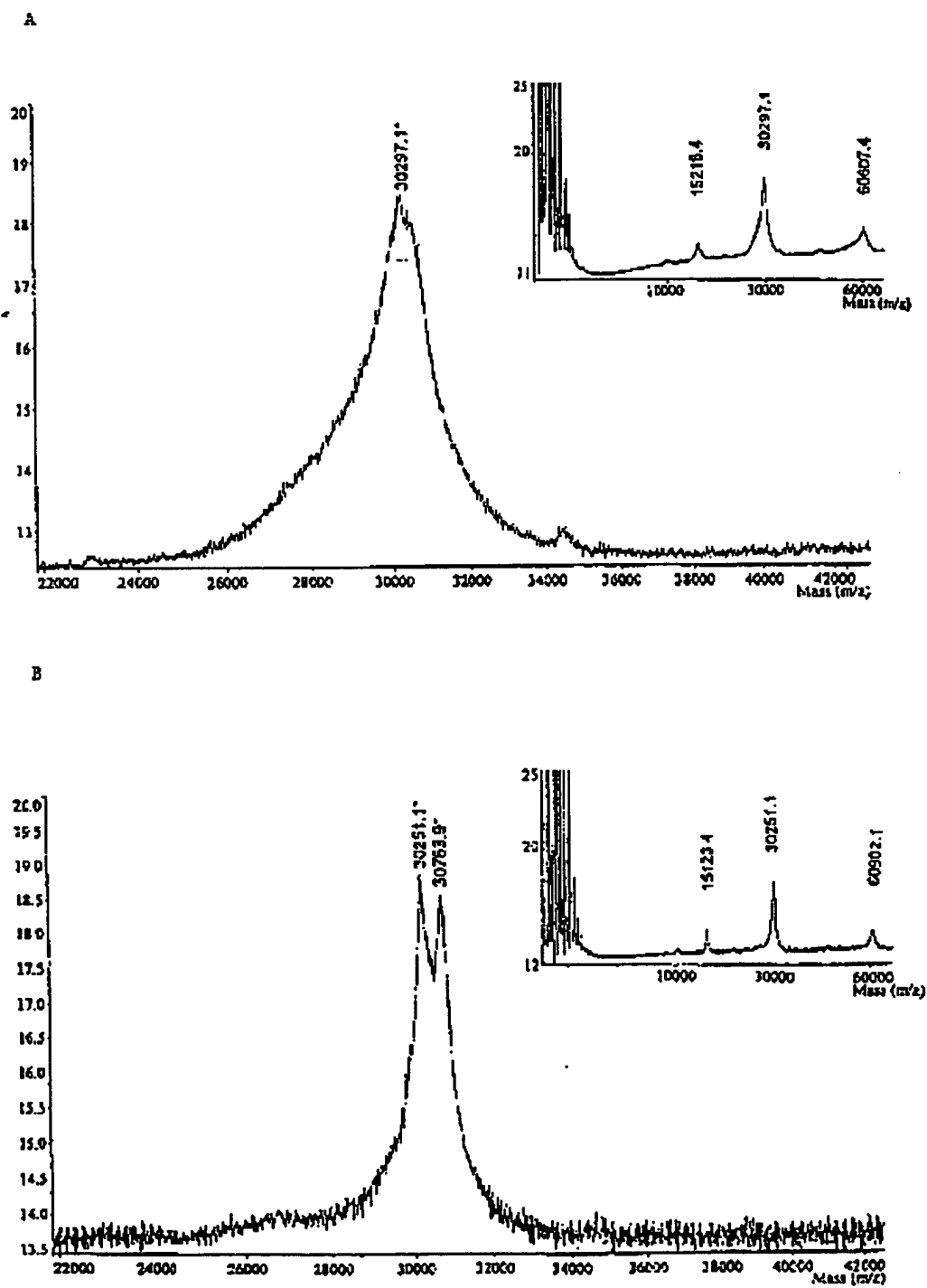
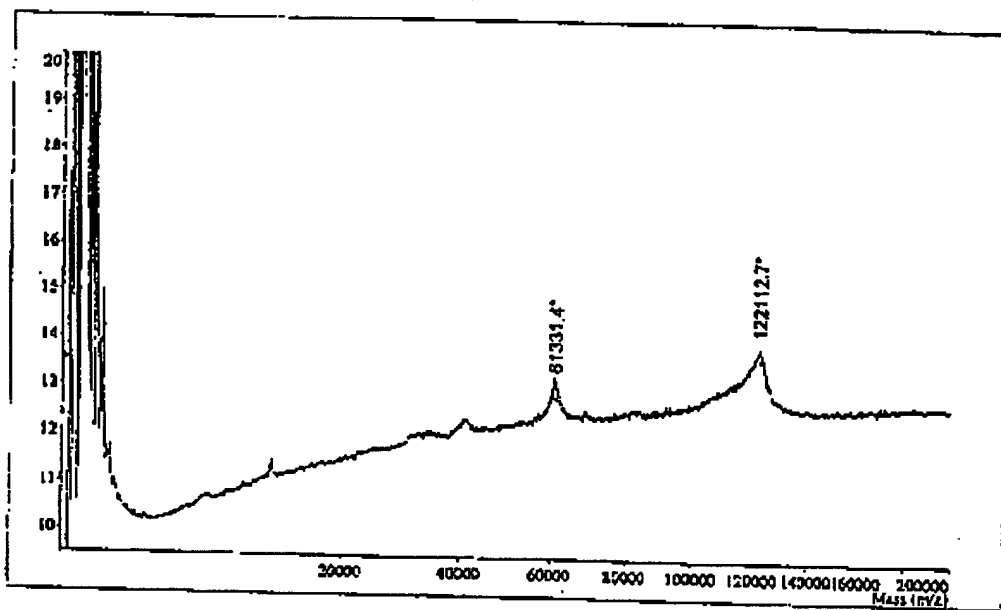


FIGURE 29

FIGURE 30

A



B

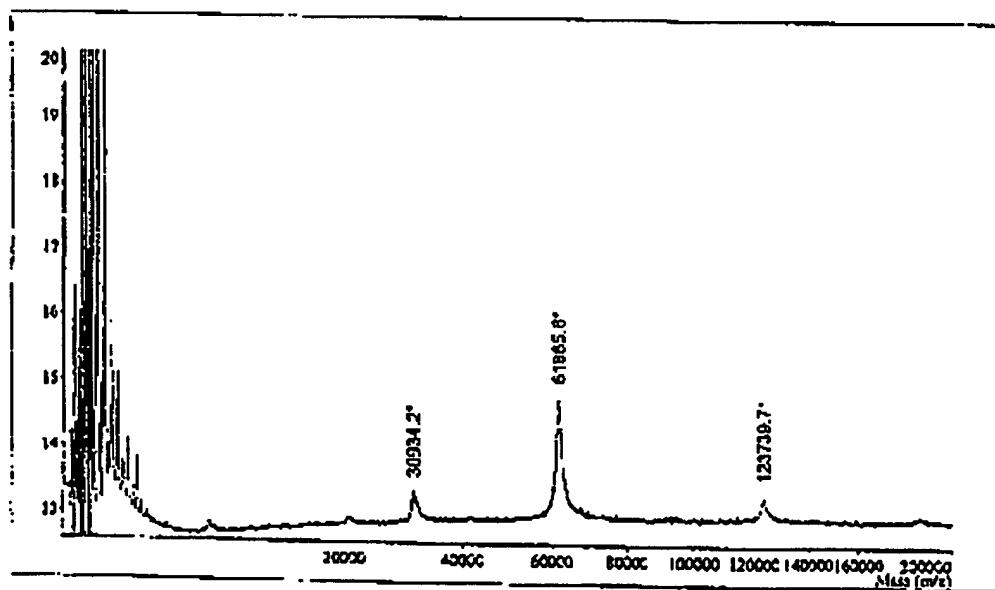
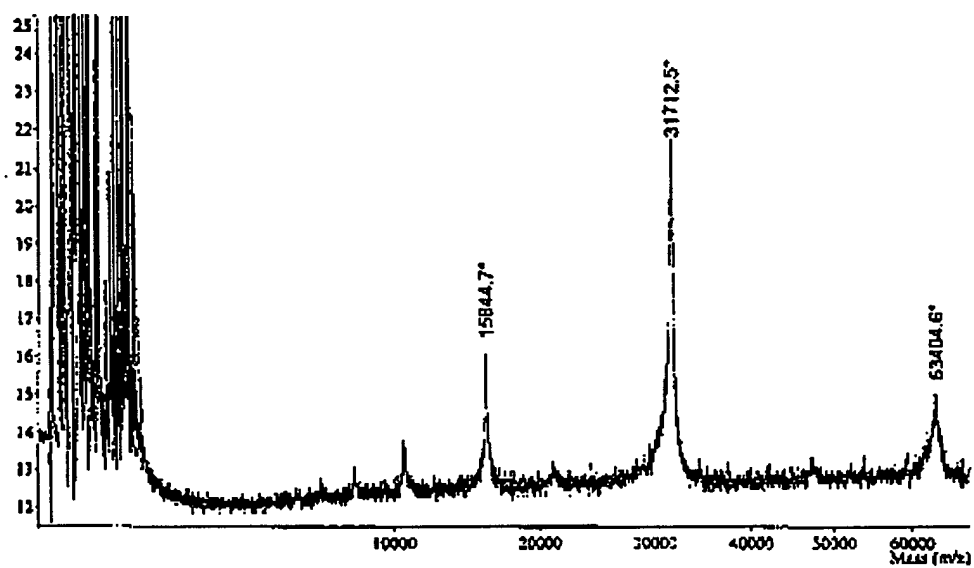


FIGURE 31

A



B

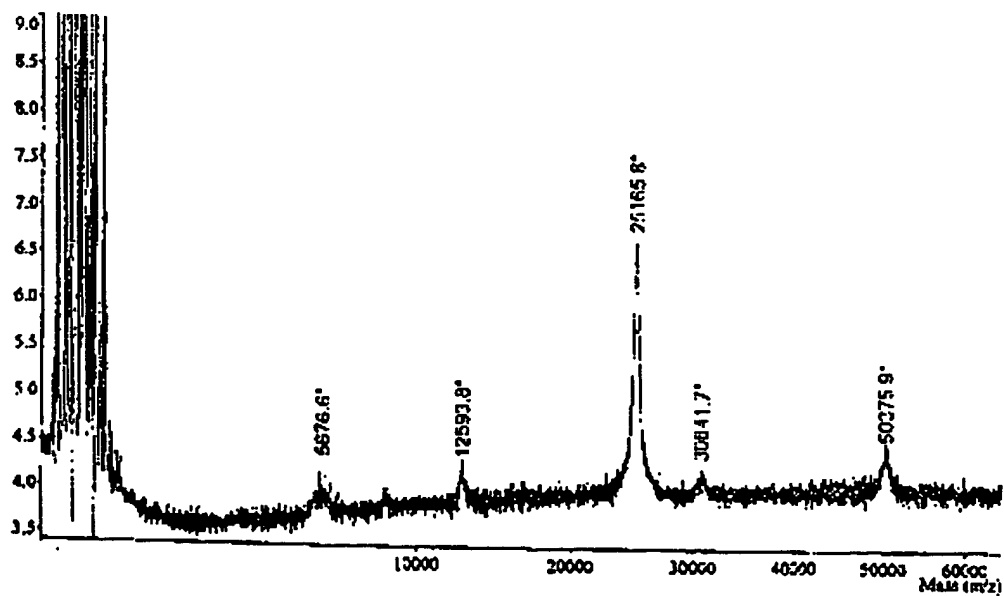


FIGURE 32A

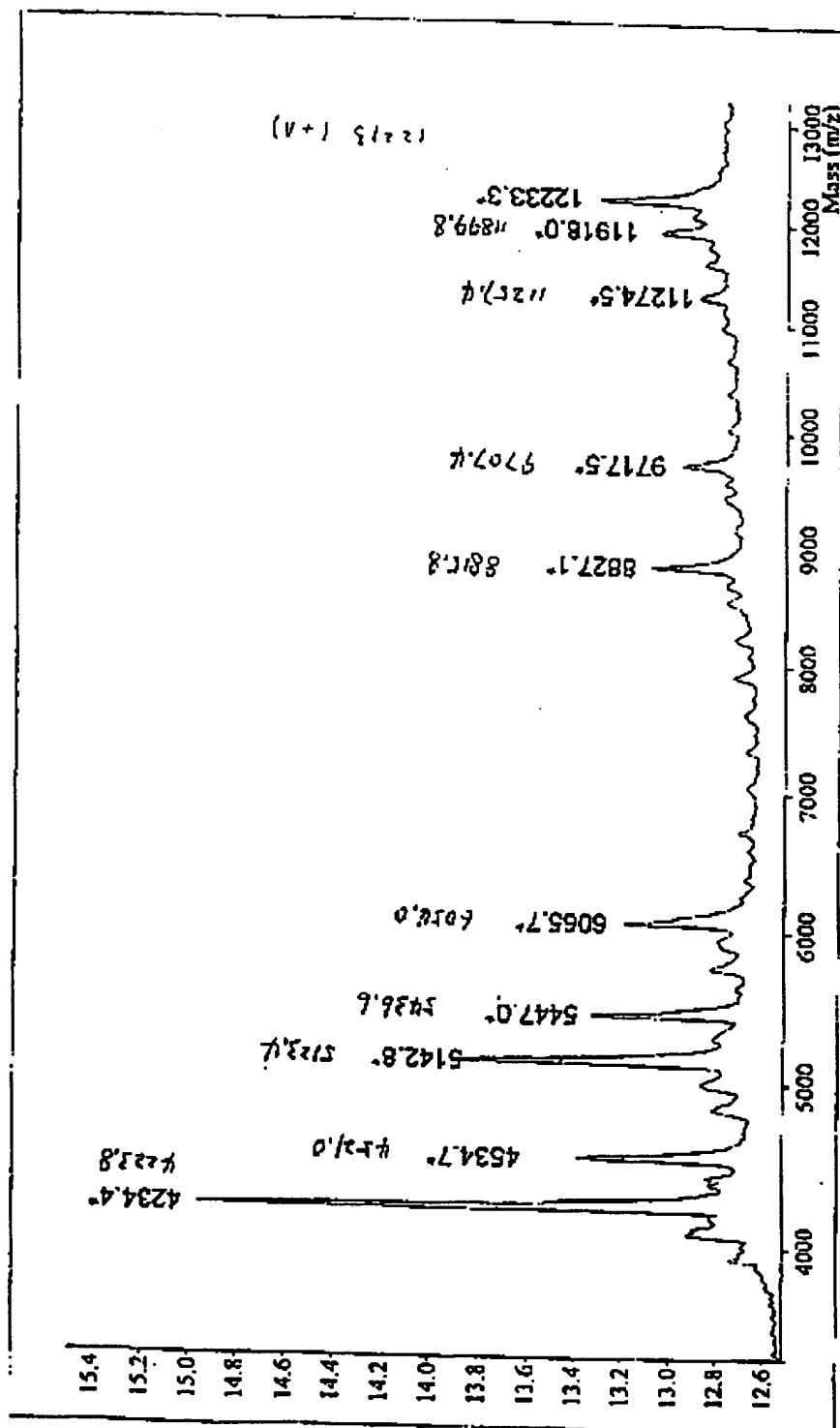


FIGURE 32B

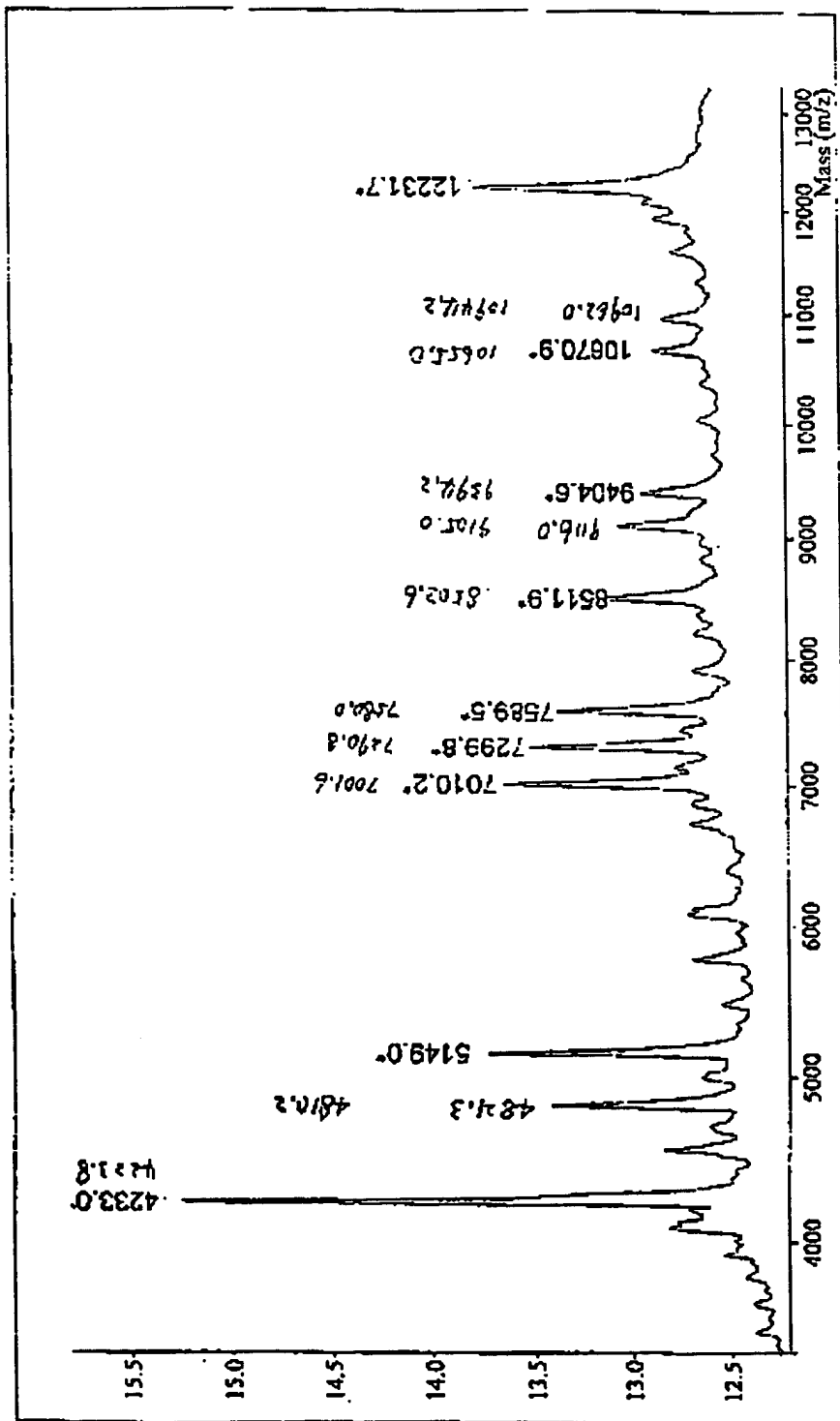


FIGURE 32C

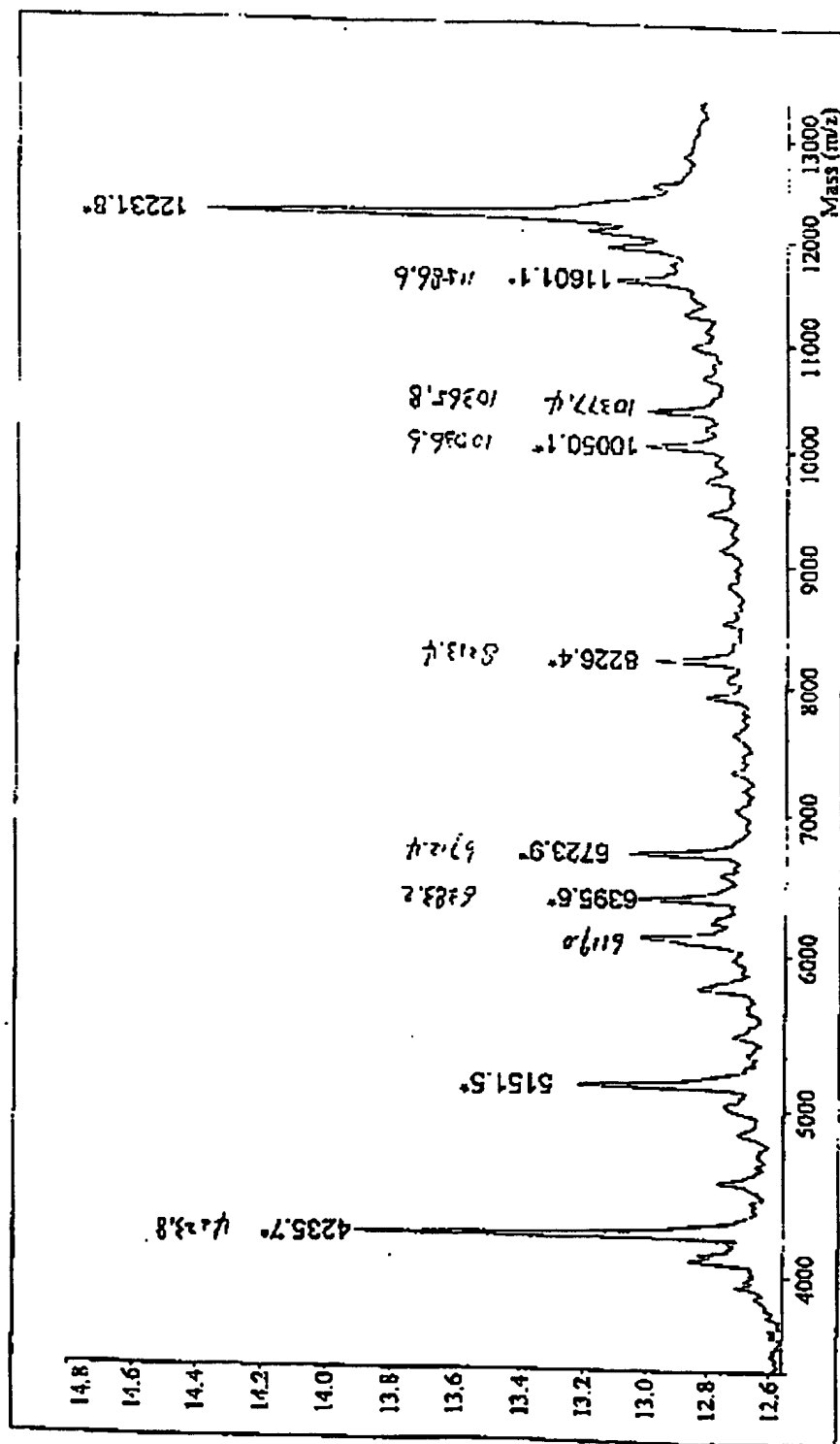


FIGURE 32D

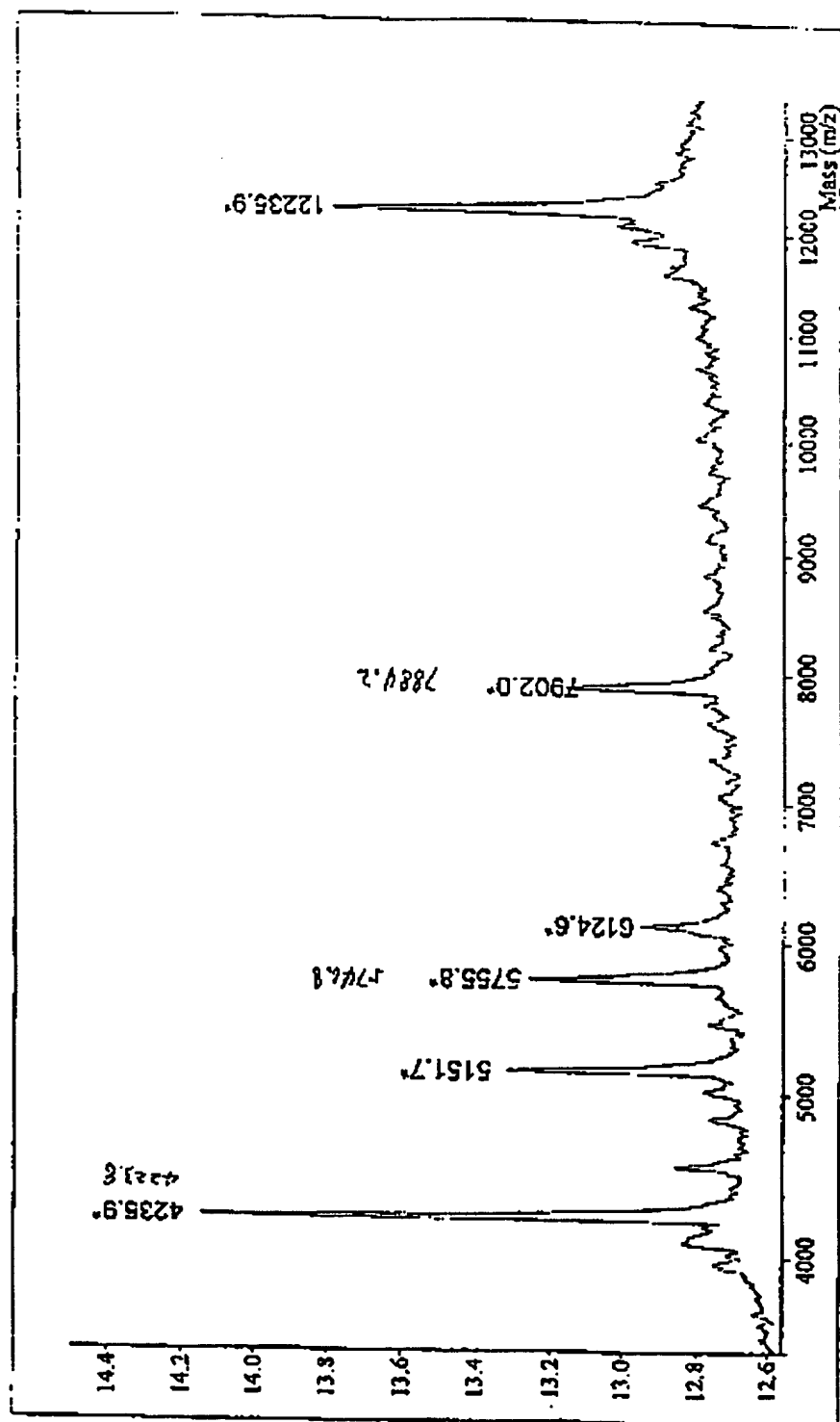


FIGURE 33

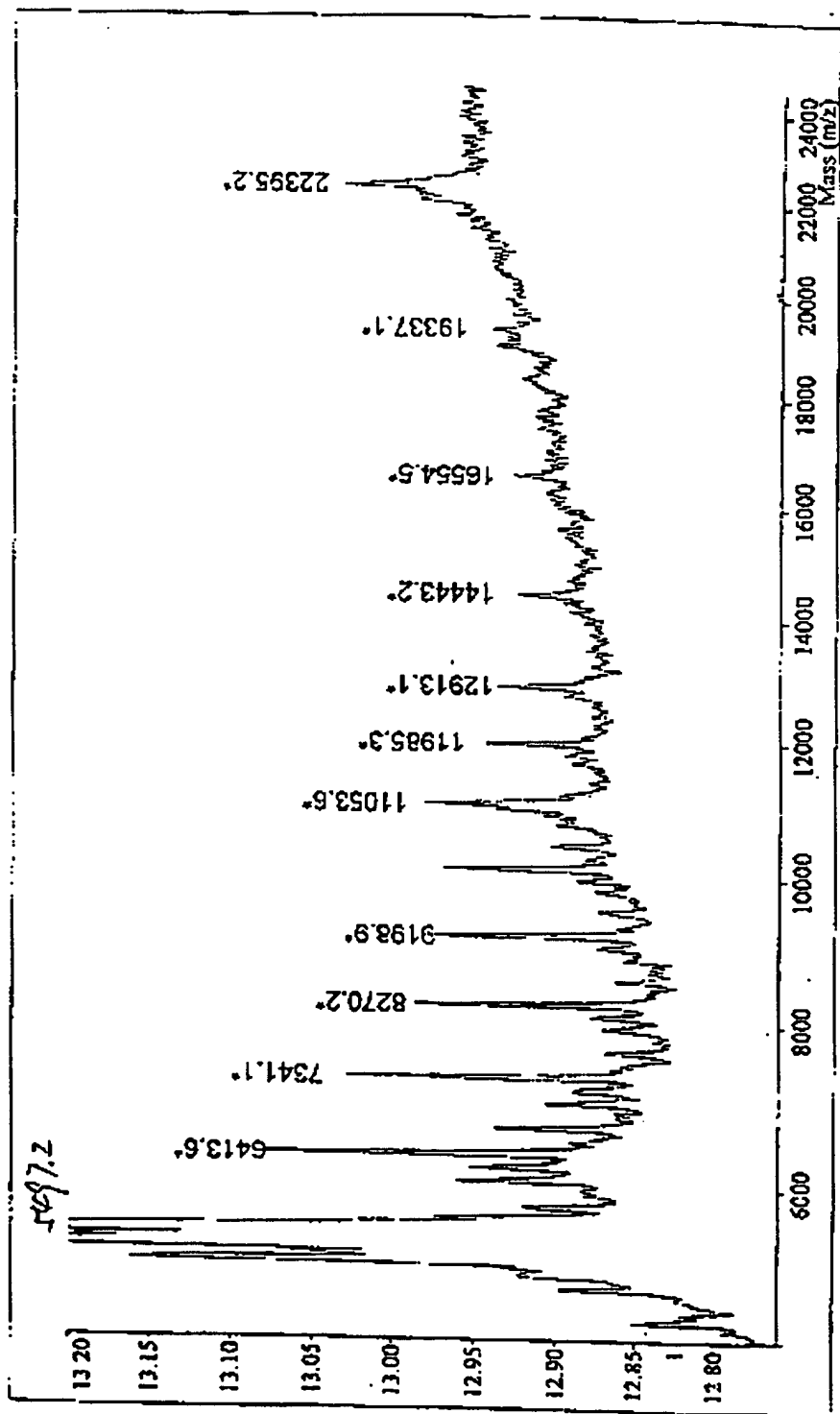
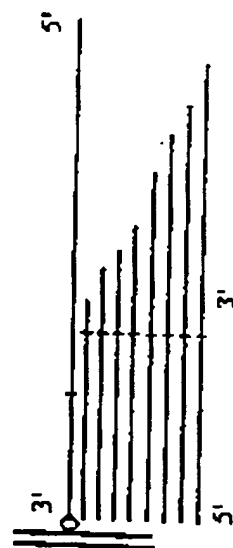
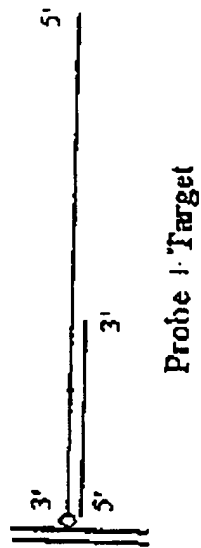
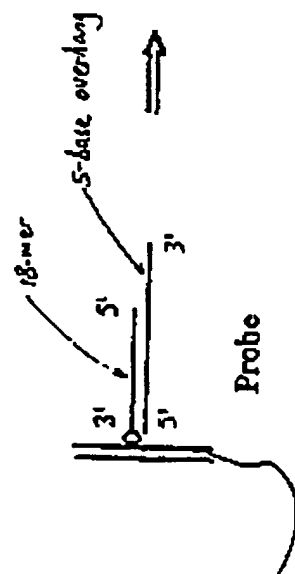


FIGURE 34



- 1). 15-mer template.
- 2). 55-mer template.

FIGURE 35A

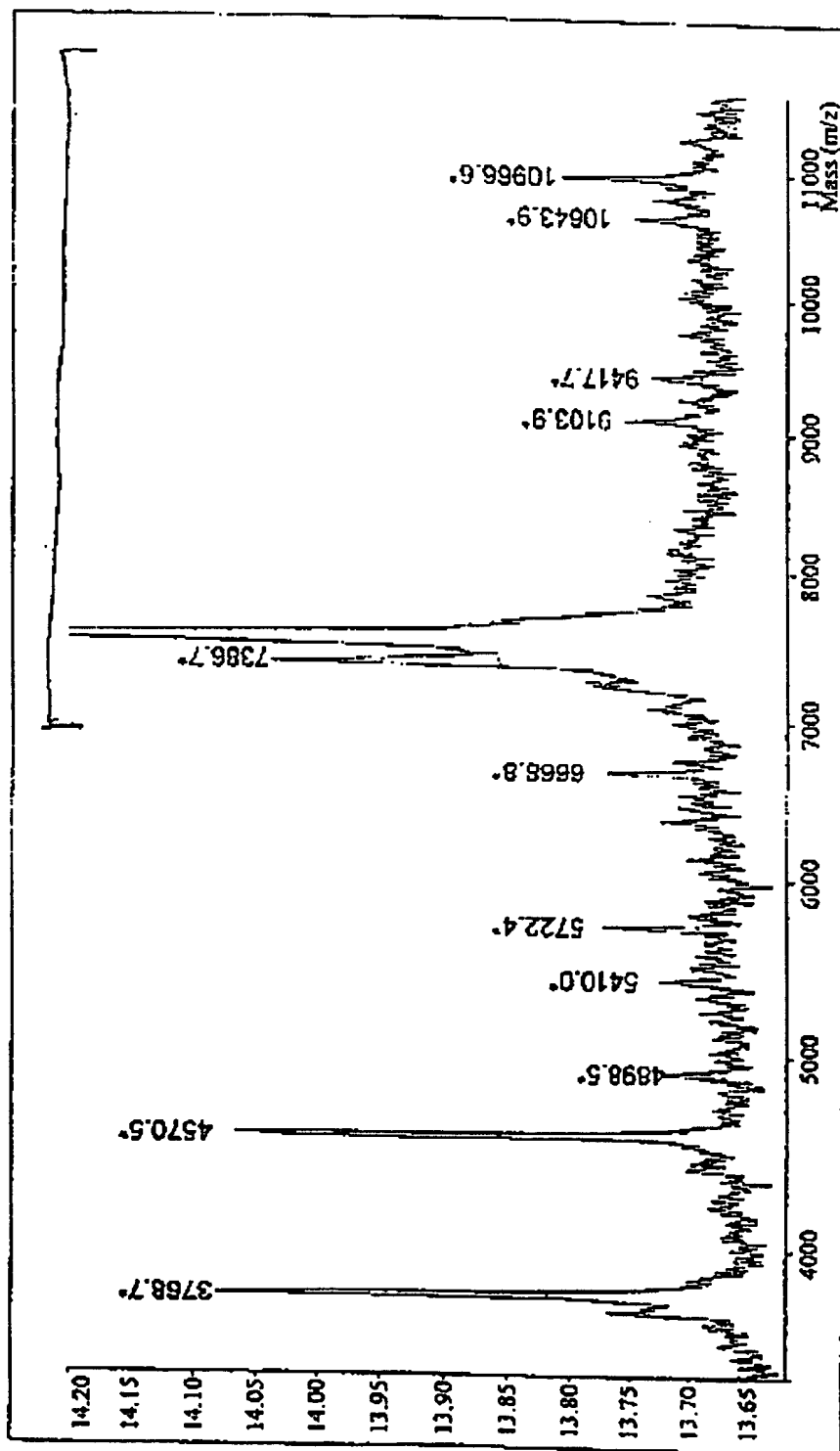


FIGURE 35B

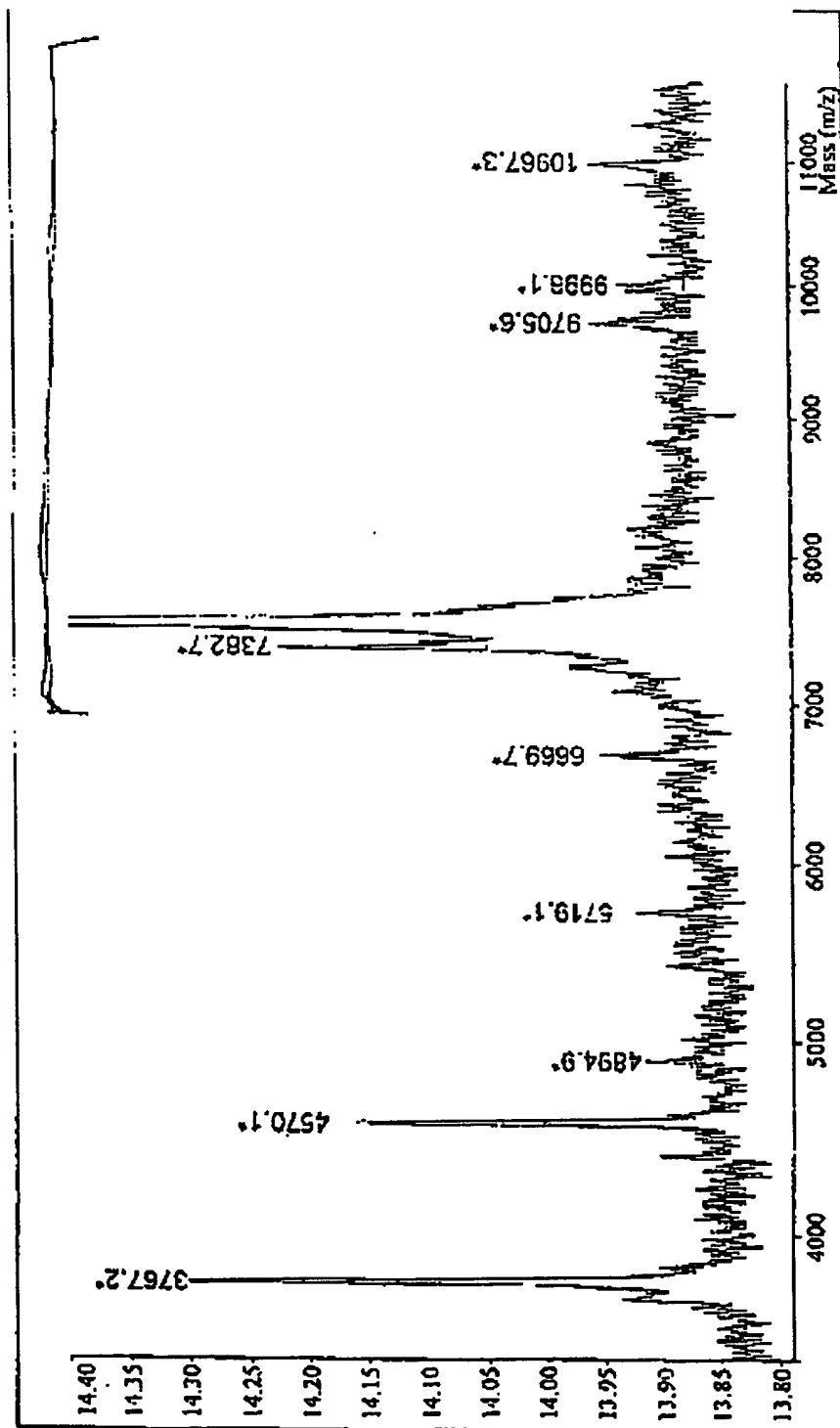


FIGURE 35C

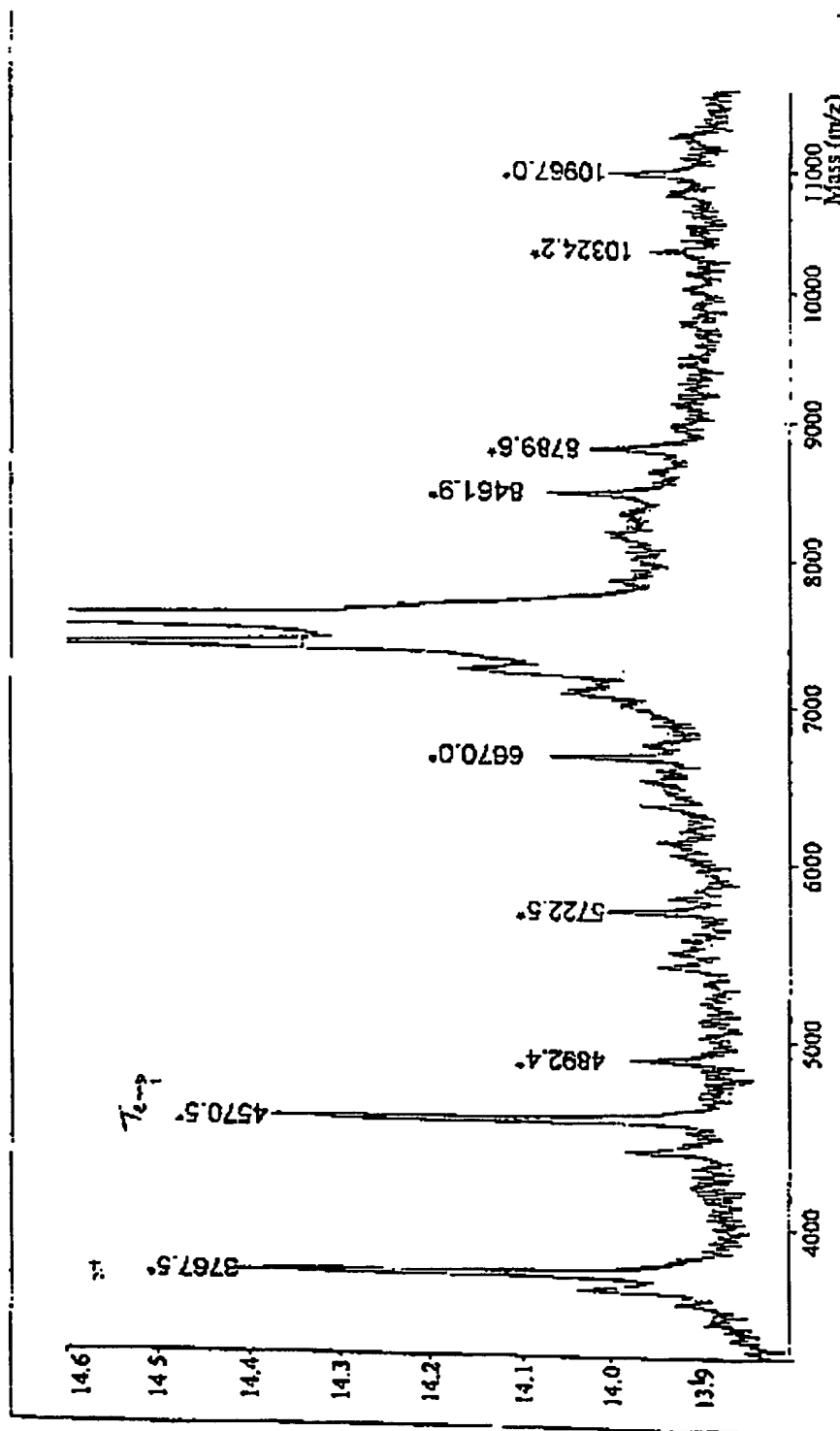


FIGURE 33D

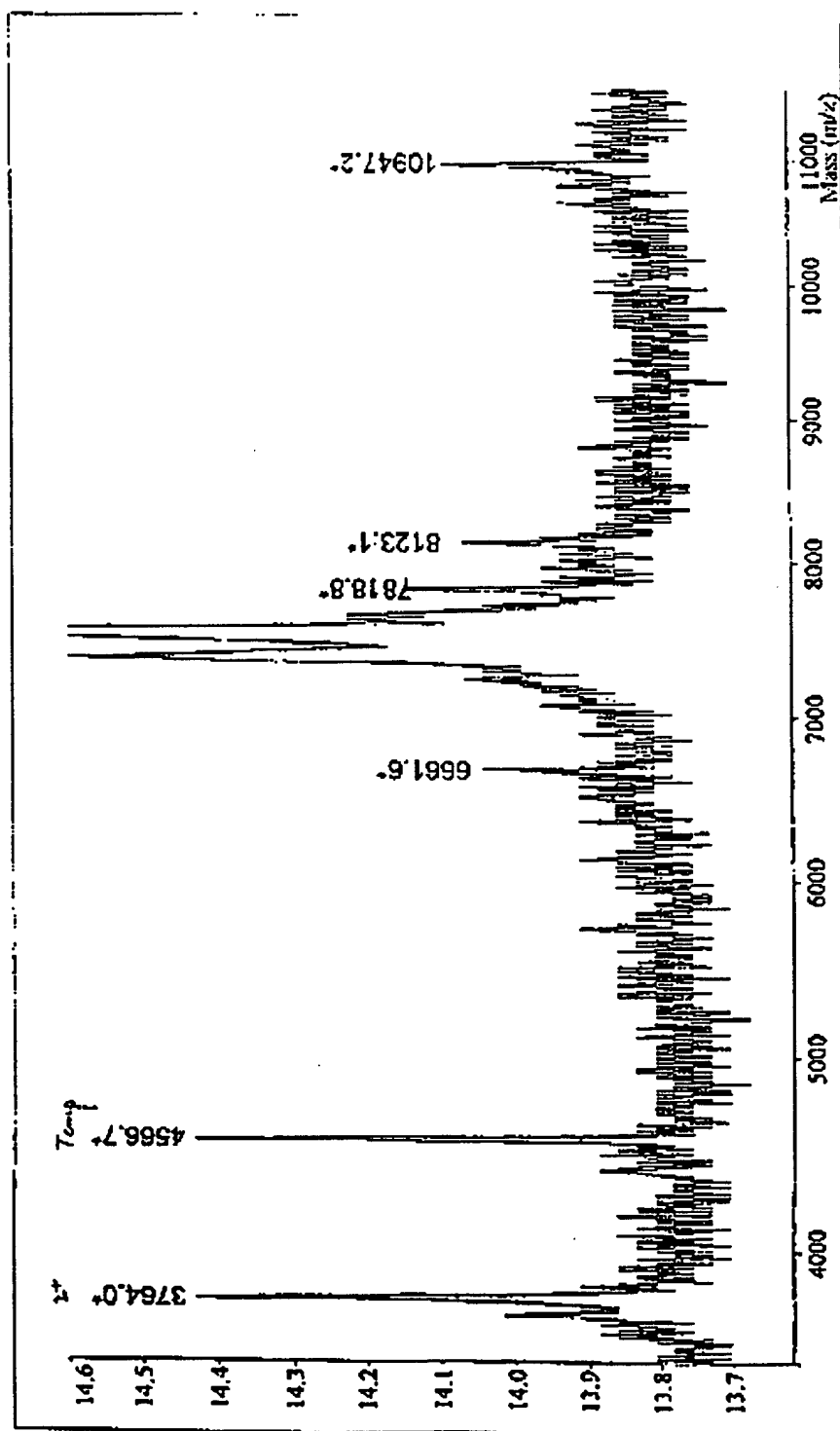


FIGURE 36

